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UNIVERSITY OF ALBERTA

OPTIMIZATION OF HUMAN ISLET ISOLATION AND CRYOPRESERVATION

BY

JONATHAN ROBERT TODD LAKEY

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degreee of **DOCTOR OF PHILOSOPHY**

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

FALL, 1995

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COMPARISON OF METHODS USED FOR THE REMOVAL OF DMSO FOLLOWING CRYOPRESERVATION AND THE DEVELOPMENT OF AN AUTOMATED PROTOCOL (submitted for publication to Cell Transplantation, 1995)

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August 24, 1995

DEDICATION

This text is dedicated to my children, Alicia, Benjamin, and Madison who I hope will enjoy the search of knowledge as much as I do; will see the world through wondering, questioning eyes; and will search for the answers they seek.

ABSTRACT

Before clinical islet transplantation can become a viable alternative in the treatment of diabetes, the consistency and reliability of human islet isolations and effective low temperature banking must be improved.

Retrospective review of 153 human islet isolations using multivariate regression analysis suggest an improved approach to the prediction of isolation success. Independent analysis of the duration of cold storage before isolation, indicated that with current methods used to isolate islets, the maximum duration of cold storage tolerated by the human pancreas is 16 hours.

Evaluation of the procurement of the pancreas specifically for islet isolation indicated that large numbers of viable islets could be recovered utilizing either an *in situ* vascular perfusion with cold University of Wisconsin solution followed by total pancreatectomy or a subtotal pancreatectomy procedure before *in situ* vascular flushing.

The ACES (automated cell extraction system) was developed as a more automated method of separating islets and incorporated a new method of tissue recombination. Experimental data from a series of canine isolations showed similar results, for the ACES system as compared with the standard system, in its effectiveness to recover islets without compromising functional viability.

Retrospective analysis of 47 preparations of banked islets showed a high proportion of microbiological contamination. Subsequent prospective evaluation of 70 consecutive human islet isolations and freezes indicated that the frequency

of contaminants decreased progressively throughout the isolation process and that islets remain sterile during low temperature storage in liquid nitrogen.

Bulk cryopreservation of isolated islets in large capacity freezer bags is a feasible option in low temperature preservation of islets. Experiments using canine islets resulted in equivalent recoveries, *in vitro* and *in vivo* function to islets cryopreserved in multiple glass tubes. A modified slow step protocol for the removal of the cryoprotectant dimethyl sulfoxide following thawing was developed for incorporation into an automated protocol using the freezer bag system. Evaluation using this automated protocol within the freezer bag resulted in equivalent recovery and functional viability as compared to standard sucrose dilution protocols. Bulk cryopreservation and low temperture storage of islets in a single freezer bag using an automated protocol is a practical and more effective alternative to cryopreservation in glass tubes.

This work has identified significant improvements in the quality and consistency of human islet isolations and improved methods for the bulk cryopreservation of islets will facilitate improved clinical islet transplant trials.

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TABLE OF CONTENTS

PAGE

I. INTRODUCTION

Diabetes 1
Pancreas Transplantation 3
Islet Transplantation 5
Methods of Islet Isolation 6
Islet Purification 10
Donor Variables 13
Pancreas Procurement 15
Whole Pancreas Preservation 16
Pancreas Preservation Before Islet Isolation 19
Islet Transplant Registry 21
Cryopreservation 22
Thesis Aims 29
References 34

II. VARIABLES IN ORGAN DONORS THAT AFFECT THE RECOVERY OF HUMAN ISLETS OF LANGERHANS

Introduction 52
Materials and Methods 53
Results 59
Discussion 63
Conclusion 69
References 78

III. HUMAN PANCREAS PRESERVATION BEFORE ISLET ISOLATION: COLD ISCHEMIC TOLERANCE

Introduction 81
Materials and Methods 82
Results 86

PAGE

Discussion 88
Conclusion 92
References 98

IV. CADAVERIC PANCREAS RECOVERY TECHNIQUE: IMPACT ON ISLET RECOVERY AND *IN VITRO* FUNCTION

Introduction 101
Materials and Methods 102
Results 107
Discussion 109
Conclusion 112
References 118

V. DEVELOPMENT OF AN AUTOMATED ISLET ISOLATION SYSTEM

Introduction 120
Materials and Methods 122
Results 129
Discussion 132
Conclusion 136
References 150

VI. MICROBIAL SURVEILLANCE OF HUMAN ISLET ISOLATION, *IN VITRO* CULTURE AND CRYOPRESERVATION

Introduction 153
Materials and Methods 154
Results 158
Discussion 161
Conclusion 165
References 171

PAGE

VII. BULK CRYOPRESERVATION OF ISOLATED ISLETS OF LANGERHANS

Introduction 173
Materials and Methods 174
Results 180
Discussion 183
Conclusion 185
References 196

VIII. COMPARISON OF METHODS USED FOR THE REMOVAL OF DMSO FOLLOWING CRYOPRESERVATION AND THE DEVELOPMENT OF AN AUTOMATED PROTOCOL

Introduction 199
Materials and Methods 200
Results 206
Discussion 208
Conclusion 212
References 223

IX. GENERAL DISCUSSION AND CONCLUSIONS

Discussion 226
Conclusions 235
References 237

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
II-1	Influence of donor characteristics on the recovery of islets isolated from human pancreases.	70
II-2	Influence of organ procurement variables on the ability to recover islets.	71
II-3	Stepwise multivariate logistic regression analysis of 19 donor related variables predicting isolation success (>100,000 IE, at a purity >50%).	72
II-4	Relationship between donor age categories and *in vitro* islet function using the calculated stimulation index.	73
II-5	Stepwise multivariate logistic regression analysis of categorized donor variables.	74
II-6	Multiple stepwise regression analysis of donor and islet isolation factors.	75
	A: Post-purification islet recovery (IE/g pancreas) as outcome variable.	
	B: Stimulation Index calculated from glucose perifusion as outcome variable.	
III-1	Islet recovery (>100,000 IE of >50% purity) and *in vitro* viability (glucose perifusion, SI >2) from cadaveric donor pancreases loaded with collagenase solution after primary pancreatectomy or pancreases flushed and stored in UW solution prior to islet isolation.	93
IV-1	Donor characteristics comparing procurement primary pancreatectomy or *in situ* vascular flushing with UW solution.	113
IV-2	Human islet recovery after primary pancreatectomy or *in situ* flush with UW solution.	114

TABLE	DESCRIPTION	PAGE
IV-3	*In vitro* viability assessment of purified islets after primary pancreatectomy or *in situ* flush with UW solution.	115
V-1	Islet losses from sluice collection system with varied collection angle and flow rate.	137
V-2	Donor and isolation variables for studies of standard vs. automated islet isolation protocol.	138
V-3	Yields of canine islets isolated with the standard or the ACES system.	139
V-4	Islet size distribution following collagenase digestion, Ficoll purification and following 24 hour *in vitro* tissue culture	140
V-5	Insulin response of isolated canine islets during static incubation.	142
V-6	*In vivo* function of alloxan-induced diabetic nude mice transplanted with canine islets isolated using either the standard isolation protocol or isolated using the ACES system.	143
VI-1	Retrospective evaluation of cryopreserved human islets (n=47) from a low temperature (-196°C) tissue bank.	166
VI-2	Transport media infection rate according to the procurement method.	167
VI-3	Microbiological surveillance of islets isolated from pancreases received with infected transport fluid.	168
VI-4	Prospective evaluation of new contaminants identified from pancreases with negative cultures in the transport media.	169
VII-1	Recovery of isolated rat islets following stepwise addition of DMSO to a final concentration of 2 M, supercooling to -7.4°C and removal of the cryoprotectant using a 0.75 M sucrose dilution.	186

TABLE	DESCRIPTION	PAGE
VII-2	Recovery of purified canine islets following cryopreservation in either a freezer bag or glass tubes.	187
VII-3	Calculated stimulation index from the glucose stimulated perifusion response of canine islets cryopreserved in either a freezer bag or in glass tubes.	188
VII-4	Survival and function of fresh and cryopreserved islet grafts transplanted under the kidney capsule of alloxan induced-diabetic Balb/c nude mice.	189
VIII-1	Islet recovery following freeze-thaw and 48 hour *in vitro* tissue culture.	213
VIII-2	Insulin response of frozen-thawed islets during static incubation	214
VIII-3	*In vivo* function of alloxan induced diabetic nude mice transplanted with frozen-thawed islets	215

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
II-1	Post-digestion (solid box) and post-purification (grey box) recoveries of islets from 153 human isolations performed over a 3 year period using an identical isolation protocol.	76
II-2	*In vitro* function of isolated islets using glucose perifusion system (mean ± SEM). Islets isolated from successful isolations (open circles) vs. islets from isolations yields < 100,000 IE (closed squares).	77
III-1	Pre-purification islet yields from human cadaveric pancreases as grouped: local pancreases loaded with collagenase solution (Local Load); local pancreases *in situ* flushed with UW solution (Local UW flush); and, distantly procured pancreases subdivided by the hours of cold storage prior to islet isolation (3-8, 8-16, and > 16 hours). Islet yield represents mean islet yield (islet equivalents per gram of processed pancreas ± SEM).	94
III-2	Post-purification islet yields from human cadaveric pancreases as grouped: local pancreases loaded with collagenase solution (Local Load); local pancreases *in situ* flushed with UW solution (Local UW flush); and, distantly procured pancreases subdivided by the hours of cold storage prior to islet isolation (3-8, 8-16, and > 16 hours). Islet yield represents mean islet yield (islet equivalents per gram of processed pancreas ± SEM).	95
III-3	Comparison of dynamic *in vitro* insulin secretion from islets isolated from local pancreases flushed with UW and processed within 3 hours (Local UW flush) (closed triangle), 3-8 hours (shaded triangle), 8-16 hours (shaded square), > 16 hours (open diamond) of cold storage prior to islet isolation as compared with islets isolated from local pancreases removed prior to UW flushing and loaded with collagenase solution (Local Load) (open circle).	96

FIGURE	DESCRIPTION	PAGE
III-4	Comparison of calculated stimulation indexes from human cadaveric pancreases as grouped: local pancreases loaded with collagenase solution (Local Load); local pancreases *in situ* flushed with UW solution (Local UW flush); and, distantly procured pancreases subdivided by the hours of cold storage prior to islet isolation (3-8, 8-16, and >16 hours).	97
IV-1	Graphic illustration of stimulated area under the curve of insulin release during *in vitro* perifusion. Pre- and post-stimulation basal insulin release is averaged and subtracted from the stimulated insulin release to yield the incremental insulin release resulting from hyperglycemic stimulus over a 60 minute period.	116
IV-2	Glucose stimulated perifusion comparing islet function from freshly isolated (upper panel) and cryopreserved islets (lower panel) isolated from pancreases procured before or after *in situ* UW flush.	117
V-1	The Automated Cell Extraction System during the recirculation phase of the islet isolation process.	144
V-2	The Automated Cell Extraction System during the dilution and collection phase of the isolation process.	145
V-3	Particle collection system used to collect digested pancreatic tissue fragments along a flat length of PVC tubing. Tissue particles are allowed to sediment and are collected into a single intravenous bag on ice. All remaining particles and the majority of the dilution media are vented into the waste port and collected in two large capacity bags.	146
V-4	Experimental protocol for the isolation of canine islets using either the standard method or using the ACES system.	147

FIGURE	DESCRIPTION	PAGE
V-5	Blood glucose profiles of alloxan-induced diabetic Balb/c nude mice receiving 2000 isolated canine islets isolated using the standard method (closed box), from the collect port of the ACES system (open circle) or from the waste port of the ACES system (open triangles). Arrows = failed transplants (blood glucose >15 mmol/L for two consecutive readings.	148
V-6	Oral glucose tolerance test of alloxan-induced diabetic nude mice transplanted with canine islets isolated using the standard isolation methods (closed box), from the collection port of the ACES system (open circles) or from the waste port of the ACES system (closed triangles) as compared with non-transplanted age matched control animals (open triangles).	149
VI-1	Schematic illustrating experimental design for prospective study of microbiological sampling following each step: 1. organ procurement and transport; 2. pancreas perfusion; 3. digestion and dissociation of pancreas; 4. purification; 5. *in vitro* islet culture; 6. cryopreservation; 7. thawing and removal of cryoprotectant; and 8. 48 hour *in vitro* culture.	170
VII-1	Temperature profiles generated by placing thermocouples in a freezer bag. Probe positioned at upper one-third of bag (open triangle), positioned in middle of bag (closed circle), positioned at bottom one-third of bag (open triangle), and placed on outside of bag (closed square)	190
	Freezer bag containing (A) 100 mL or (B) 200 mL of 2 M DMSO solution, supercooled for 5 minutes before attempting to nucleate or (C) freezer bag containing 100 mL of 2 M DMSO solutin with cryopreservation protocol modified to allow 10 minutes of supercooling at -7.4°C prior to nucleation.	

FIGURE	DESCRIPTION	PAGE
VII-2	Temperature profile generated with a single thermocouple placed within a single glass freezer tube (closed square) and subjected to the standard islet cryopreservation protocol. The second probe (open triangle) was placed on the outside of the tube as a reference.	191
VII-3	Temperature profile from a freezer bag containing three thermocouples and 100 mL of 2 M DMSO solution during slow cooling at 0.25°C/min from -10°C to -40°C. Probe positioned at upper one-third of bag (open traiangle), positioned in middle of bag (closed circle), positioned at bottom one-third of bag (open triangle), and placed on outside of bag (closed square).	192
VII-4	Temperature profiles generated from a freezer bag containing three thermocouples and 100 mL of 2M DMSO solution during rapid thawing from -196°C to 0°C in a 40°C water bath with a calculated thawing rate of 175-200°C/minute. Probe positioned at upper one-third of bag (open triangle), positioned in middle of bag (open circle), positioned at bottom one-third of bag (closed square)	193
VII-5	Glucose stimulated perifusion response of canine islets either cryopreserved in a freezer bag (closed diamonds) or in glass tubes (open triangles). Non-frozen canine islets (closed squares) from the same islet isolation are included as controls.	194
VII-6	Non-fasting blood glucose profiles of alloxan-induced diabetic nude mice receiving 2000 frozen-thawed canine islets cryopreserved in a freezer bag (open circles) or glass tubes (shaded box). Non-frozen canine islets (closed diamonds) are included as controls for comparative purposes. At 30 days post-transplant removal of the islet graft resulted in prompt hyperglycemia. Arrow = failed transplant.	195
VIII-1	Protocol for the rapid thawing and removal of the cryoprotectant using a 30 minute exposure to 0.75 M sucrose solution followed by serial dilution with Thaw Media.	217

FIGURE	DESCRIPTION	PAGE
VIII-2	Protocol for the thawing and removal of the cryoprotectant using slow step dilution.	217
VIII-3	Protocol for the thawing and removal of the cryoprotectant using a modified slow step dilution.	218
VIII-4	Oral glucose tolerance test of alloxan-induced nude mice transplanted with canine islets and monitored for 50 days.	
	A. Mice receiving freshly isolated islets (open triangle), or islets cryopreserved in glass tubes with the DMSO removed using sucrose dilution (closed box), standard slow step (closed circle) or the modified slow step protocol (closed diamond).	
	B. Mice receiving freshly isolated islets (open triangle), or islets cryopreserved in freezer bags with the DMSO removed using sucrose dilution (closed box) or modified slow step protocol (closed diamond).	219

LIST OF PHOTOGRAPHIC PLATES

PLATE	DESCRIPTION	PAGE
Plate VIII-1:	Photomicrograph of freshly isolated canine islets placed under the renal capsule of an alloxan-induced diabetic nude mouse. Aldehyde-fuchsin stained section showing well granulated beta cells. (original magnification x 200)	220
Plate VIII-2:	Photomicrograph of frozen-thawed canine islets cryopreserved in the freezer bag and the DMSO removed using the standard sucrose dilution protocol. Islets were placed under the renal capsule of an alloxan-induced diabetic nude mouse. Aldehyde-fuchsin stained section showing well granulated beta cells. (original magnification x 200)	221
Plate VIII-3:	Photomicrograph of frozen-thawed canine islets cryopreserved in the freezer bag and the DMSO removed using the modified slow step protocol. Islets were placed under the renal capsule of an alloxan-induced diabetic nude mouse. Aldehyde-fuchsin stained section showing well granulated beta cells. (original magnification x 200)	222

LIST OF ABBREVIATIONS

ACES	automated cell extraction system
ALS	antilymphocyte serum
ATG	antithymocyte globulin
AUC	area under the curve
β	beta
BMI	body mass index
BSA	bovine serum albumin
CDD	continuous digestion device
CPP	cryoprecipitated plasma
DCCT	Diabetes Control and Complications Trial
DMSO	dimethyl sulfoxide
EGTA	ethyleneglycol-bis-(b-aminoethyl) N,N^1-tetraacetic acid
FCS	fetal calf serum
HBSS	Hanks' balanced salt solution
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid
HLA	human leukocyte antigen
HOC	hyperosmolar citrate
IBMX	3-Isobutyl-1-methylxanthine
IDDM	insulin-dependent diabetes mellitus
IE	islet equivalent to 150 μm in mean diamter
MHC	major histocompatibility complex
MST	median survival time
NCS	newborn calf serum
NIDDM	non-insulin-dependent diabetes mellitus
OGTT	oral glucose tolerance test
OR	odds ratio
P/S	penicillin/streptomycin
RPMI	Roswell Park Memorial Institute
SEM	standard error of the mean

LIST OF ABBREVIATIONS - continued

SGF	silica gel filtered
SI	stimulation index
STZ	streptozotocin
UW	University of Wisconsin
VAF	virus antigen free

I

INTRODUCTION

DIABETES

Nearly eleven million North Americans are afflicted with diabetes mellitus, a chronic disorder of glucose and carbohydrate metabolism (1, 2). This devastating disease is characterized by various physiological abnormalities, most notably, prolonged hyperglycemia (3). Defective biosynthesis, release or utilization of the pancreatic hormone, insulin, results in hyperglycemia. The prolonged exposure to hyperglycemia can have a profound and devastating impact on the metabolism, resulting in nephropathy, retinopathy, neuropathy and angiopathy (4, 5). These chronic complications are manifested in diabetic persons as the following increased risks: ten times more coronary artery disease with atherosclerosis appearing at an early age; four to six times more cataracts, twenty-five times more blindness; seventeen times more kidney dysfunction; one half of which progress to renal failure as a result of nodular glomerulosclerosis; sixteen times more gangrene of the limbs leading to amputations; sensory and autonomic changes due to neuropathic deterioration (5, 6-8). Consequently, the life expectancy of those individuals afflicted with diabetes is only two-thirds that of the normal population (1, 2). Diabetes is the fifth leading cause of death in North America accounting for 15.2 deaths per year per 100,000 deaths (1, 8). Despite improvements in the treatment of diabetes, the estimated annual costs for health care and lost wages in the United States is approximately 92 billion dollars (9).

Diabetes can be classified as primary or secondary. Type I, primary juvenile diabetes or insulin-dependent diabetes mellitus (IDDM), is characterized by the abrupt onset of symptoms, primarily before the age of 25 years and most notably absolute insulin deficiency (4, 8). Type II primary diabetes or non-insulin-dependent diabetes mellitus (NIDDM) occurs predominately in mid or late life and is prevalent in obese individuals and is related to insulin resistance (10). Treatment of type II diabetes is normally managed with a balance of energy expenditure and strict dietary control (4). Secondary diabetes mellitus can result from chronic pancreatitis, hormonal abnormalities, and certain genetic syndromes (10, 11).

Before the discovery of insulin by Banting and Best in 1922, the life expectancy of type I diabetics was bleak, with many patients dying within one year of diagnosis (12). Wide fluctuations of hyperglycemia in type I diabetics are controlled through balancing dietary intake and energy expenditure with exogenous insulin therapy. The exogenous administration of insulin is required in order to maintain the fasting plasma glucose concentrations within normal ranges. However, stringent control and intensive multiple daily insulin injections are needed to maintain normoglycemia (13, 14).

The Diabetes Control and Complications Trial Research Group (DCCT) recently published their findings of a multi-centre trial where type I diabetic patients were given intensive insulin therapy with the aim of maintaining blood glucose concentrations close to the normal range to determine if the frequency and severity of the secondary complications of diabetes could be reduced (15). Longterm intensive insulin therapy in conjunction with increased medical management delayed

the onset or slowed the progression of retinopathy, neuropathy, and nephropathy (15, 16). However, in another study, the benefits of intensive insulin therapy were coupled with substantial risks and costs including a three-fold increase in hypoglycemic episodes, increased body weight gain and an increased incidence of local infections (17). A physiologic alternative to exogenous insulin therapy for patients with IDDM, supported by the findings of the DCCT, is "total endocrine replacement therapy" via transplantation of the intact pancreas or transplantation of a free graft of pancreatic tissue (18-20). Transplantation of the intact pancreas involves either a vascularized whole organ or a segmental portion of the pancreas, whereas, transplantation of a free graft of pancreatic tissue involves either dispersed pancreatic tissue fragments or isolated islets of Langerhans.

PANCREAS TRANSPLANTATION

Early efforts to alleviate diabetes through pancreas transplantation were attempted soon after the work of Von Mering and Minkowski, who in 1889, were the first to show that hyperglycemia developed following the complete removal of the canine pancreas (21-23). The first clinical attempt at vascularized pancreas transplantation was performed in 1966 by Drs. William Kelly, Richard Lillehei and associates at the University of Minnesota (24). One of the major obstacles to the success of pancreas transplants, was the management of the digestive enzymes secreted by the exocrine portion of the pancreas. Anastomosis of the pancreatic duct in the transplanted pancreas to the ureter (25, 26), jejunum (27) and free drainage into the peritoneal cavity (28) were attempts made to control exocrine secretions and

to prevent autodigestion and eventually graft failure (25, 29). Blockage of the pancreatic duct with neoprene (30) or ligation of the pancreatic duct (31) have also been attempted to manage or control the exocrine drainage (20, 25). However, none of these techniques used for management of exocrine drainage yielded satisfactory results and the morbidity and mortality following transplantation was unacceptably high (32).

Drainage of the pancreatic duct into the bladder was developed as an alternative method of managing the exocrine secretions (32-34). This technique offered simplicity, low infection rate and low incidence of surgical complications and the ability to monitor graft function by measuring urinary amylase (32). Advancements in immunosuppressive drug therapy and better management of the pancreatic duct have allowed a rapid increase in the number of centres performing clinical pancreas transplantation. To May 1995, there have been 6856 pancreas transplants worldwide reported to the International Pancreas Transplant Registry (35, 36). Both patient and graft functional survival rates have progressively improved with time. The current one year patient survival rate is 91% and the one year insulin independent rate is 74% in patients receiving a kidney-pancreas transplant (35).

Patients with successful pancreas transplants have normal levels of glycosylated hemoglobin (Hb A1c) (12) and normalized intravenous glucose tolerance (37). The effect on the secondary complications include a decrease in the progression of diabetic glomerulopathy (38) with a decrease in renal glomerular mesangial volume from biopsies taken at 2-3.5 years post-transplant in the simultaneously transplanted kidney (39). A beneficial effect on autonomic neuropathy following pancreas

transplant has been detected with either an improvement or stabilization of sensory, motor and autonomic indices (40). Quality of life and assessment of functional disabilities using a sickness impact profile score also showed improvements following pancreas transplantation (41). The progression of retinopathy was not improved following pancreas transplantation with approximately 30% advancement to a higher grade of retinopathy at 3 years post-transplant (42).

Technical difficulties associated with the pancreas transplantation procedure, the surgical complexity of the procedure, the requirements for and complications of lifelong immunosuppression, the incidence of post-surgical complications and the failure to improve retinopathy have led researchers to explore a much simpler approach to restoring euglycemia; transplantation of isolated islets of Langerhans.

ISLET TRANSPLANTATION

The option of transplanting only the endocrine portion of the pancreas has several theoretical advantages over pancreas transplantation. Islet transplantation is a less complicated surgical procedure than pancreas transplantation and the problems associated with management of the exocrine secretions are nonexistent (20). Post-transplant complications and mortality associated with the transplantation of only the endocrine portion of the pancreas is considerably reduced as compared to whole pancreas transplantation. Although one of the theoretical advantages of islet over pancreas transplantation is the reduced transplantable mass being less immunogenic, it has been shown that islet grafts are as immunogenic as whole pancreas grafts (43, 44). However, islet grafts can be altered or modulated to reduce

their immunogenicity. This can be achieved by a number of techniques including room temperature culture (22°C for 7 to 10 days) (45), high oxygen exposure (46), UV irradiation (47), low pH culture (48), cryopreservation (49) and by the use of antibodies that mask major histocompatibility complex (MHC) Class II antigens and complement (50, 51). The ability to use encapsulation, either micro (52) or macro (53), where the islets can be placed in a semipermeable membrane and protected from immune response, is another potential advantage of using a cellular graft over whole pancreas transplantation. In an attempt to protect islet grafts from immunological destruction, a number of immunologically privileged sites have been attempted, including: the anterior chamber of the eye (54), the cheek pouch (55), the brain (56, 57) and the testis (58). These advantages clearly indicate the potential therapy provided by transplantation of isolated islets. Critical to the success of islet transplantation as a viable approach in the treatment of diabetes is the ability to successfully isolate islets of Langerhans while maintaining viability. Researchers are continuing to attempt to improve the techniques for the isolation of islets which have evolved over the past 85 years.

METHODS OF ISLET ISOLATION

The basic principles involved in the isolation of human islets have evolved from those developed for the isolation of rodent and canine islets. In 1911, Bensley was the first to extract islets of Langerhans by hand picking rodent islets following staining of the pancreatic tissue with neutral red stain (59, 60). Hellerström developed a method of free-hand microdissection, mechanically removing the islets

from the surrounding tissue via a dissecting microscope (61). This was a labour-intensive technique with a poor overall recovery of islets.

A significant advance was made by Moskalewski who, in 1965, introduced the use of the enzyme collagenase, a compound produced by fermentation of the bacteria, Clostridium histolyticum, to isolate functionally viable islets of Langerhans from the surrounding tissue of the guinea pig pancreas (62).

This enzyme blend which contains several proteolytic enzymes, cleaves the peptide bonds in the collagen fibres of the pancreatic tissue and cleaves the connective tissue adhesions that bind the islet to the exocrine tissue (63, 64). The effective separation of the islet from the exocrine tissue is dependent on an effective lot of collagenase. One of the major barriers in clinical transplantation is the lot to lot variation of the collagenase enzyme. Furthermore, a collagenase preparation that is suitable for the digestion of the pancreas in one animal model may be ineffective when attempted in another animal model. Consequently, the selection of an adequate collagenase preparation is a matter of trial and error. Key investigations are analyzing the specific and active chemical components of crude collagenase preparation in hopes that this will one day lead to the formulation of an enzyme blend that is specific and reproducible, allowing the effective separation of large numbers of viable islets (63).

The next advancement in the isolation of rodent islets was when Lacy and Kostianovsky distended the pancreas by retrograde perfusion via the common bile duct with Hanks' balanced salt solution (HBSS) (65). The intralobular distension provided a means to mechanically separate the islets from the exocrine tissue before

mechanical mincing and enzymatic digestion. This enabled the recovery of two to three hundred islets from a single rat pancreas (65).

Extrapolation of the pancreas dissociation techniques, developed using the rodent model, to canine and human pancreases was very difficult given the compact and fibrous nature of the pancreas (46). Introduction of collagenase into the pancreas via the main pancreatic duct provided a means to uniformly distribute the enzyme throughout the exocrine tissue allowing specific enzymatic cleavage of the islets from the surrounding connective tissue. Using the ductal delivery approach large numbers of islets could be isolated from the canine pancreas (66, 67). Further modifications of this technique allowed the recovery of islets from the human (68, 69) monkey (70), and the pig pancreas (71).

Delivery of the enzyme can be accomplished with either a single injection approach using a syringe (72) or by retrograde perfusion. Horaguchi and Merrell were the first to demonstrate the delivery by retrograde perfusion and were able to recover approximately 57% of the islet mass (66). Rajotte and Warnock introduced a stainless steel recirculating perfusion device to deliver the collagenase solution to the connective tissue at the islet-acinar interface under controlled pressures (73-76). This perfusion device allowed delivery of the collagenase under controlled temperatures and pressures. Comparisons showed that loading the enzyme using the mechanical device provided superior results over the much simpler approach of delivery using a single injection (75, 76). However, because of the increased costs associated with the manufacturing of a perfusion device and the increased volumes of collagenase required, most islet centres use the simple single injection technique.

Once the enzyme is loaded into the pancreas, the next process in the isolation of islets is the mechanical separation of the islets from the exocrine tissue. The tissue chopping techniques developed in the rodents were ineffective with canine and human pancreases (77). Researchers have attempted a number of other techniques including the use of tissue macerators (78), counter-rotational blade screens (79, 80) and Velcro (81) to separate partially digested islets from the unwanted exocrine tissue. However, the shearing forces created using these methods caused excessive islet fragmentation (68, 82).

Gray et al. described a more gentle approach, manual titruation, where islets could be effectively separated from the undigested fibrous capsule by gently teasing the gland apart and shaking the tissue with forceps (68). Partially collagenase-digested tissue was then passed through different sized needles until the islets were free from the exocrine tissue (68). The next advancement in the field of islet isolation was the introduction of a continuous digestion device (CDD) by Ricordi et al. (83). This closed chamber recirculating system allowed for the collection of islets as they were progressively liberated from the digestion chamber. The collagenase distended pancreas is placed inside the stainless steel chamber that is separated by a stainless steel screen. The pancreas is mechanically dissociated by a shaking motion with the addition of glass marbles within the chamber to assist with dissociation (83). Tissue samples can be periodically removed and evaluated to determine the end point of the digestion process and to prevent overdigestion and fragmentation of the islets. Both the manual titruation and the automated dissociation (CDD) protocols have been used in the isolation of human islets. A

comparative study between the manual and automated techniques has demonstrated that the automated technique with a gentle shaking approach provided improved islet recoveries from the canine pancreas (84).

ISLET PURIFICATION

Transplantation of unpurified pancreatic tissue into patients with type I diabetes was pursued by several investigators in the 1980s in an attempt to maximize the number of islets being transplanted (77). The post-surgical complications associated with intraportal transplantation of unpurified tissue included: disseminated intravascular coagulation; systemic hypotension and portal hypertension; leading to hepatic necrosis and death (85-88). This suggests that some form of islet purification may be required. Although the purification of islets is not essential, there are several advantages to transplantation of highly purified islets. These include: improved islet engraftment (89, 90), reduced immunogenicity (91) and increased safety (92, 93).

One approach to islet purification is the separation of islets from the tissue digest based on differences between the size of the islets and the exocrine tissue. This approach has been attempted using sieves (94), sedimentation at unit gravity (62, 65), centrifugal elutriation (95, 96) and by isokinetic gradient centrifugation (97). However, using these methods islets which are the same size as the exocrine tissue cannot be separated. Other approaches to islet purification include the use of islet- or exocrine-specific lectins or antibodies (98-101). Once attached, these particles can be bound to magnetic microspheres and purified based on magnetic extraction.

Bound particles can also be subjected to laser photodynamic therapy (102). The observation that islets are less radiosensitive than exocrine tissue led to a purification approach where pancreatic microfragments were subjected to radiation (103).

Despite a significant amount of effort in alternative purification methods, the most widely used approach to islet purification is density gradient purification. Density dependent or isopycnic separation of tissue is based on the fact that cells will migrate and settle during centrifugation to the location in the gradient where their density is equal to that of the density gradient solution (93). The effectiveness of this purification technique relies on differences in the density between islets and exocrine tissue. Lacy and Kostianovsky attempted separation of rodent islets from the exocrine tissue by centrifugation in a discontinuous sucrose gradient (65). The hyperosmolar sucrose solution was shown to affect the *in vitro* response of the isolated islets and was replaced with Ficoll, a high molecular weight (400,000 dalton) polymer of sucrose, by Lindall et al., in 1969 (104). Scharp et al. (1973) dialysed the Ficoll before centrifugation to remove contaminants from the Ficoll solution, further improving islet recovery (105). Islets isolated with this collagenase digestion method and purified using Ficoll gradients responded to glucose stimulation similar to those isolated using hand picking (106).

When using the density gradient centrifugation approach to purify islets, a number of other density solutions have been attempted including: bovine serum albumin (BSA) (107, 108), dextran (109), hypaque-Ficoll (110), metrizamide (111), percoll (112) and diatrizoate sodium (113). In 1987, Euro-Collins, a cold storage preservation solution was used as the vehicle for dissolving the Ficoll powder (114).

It has been shown that hypertonic density solutions such as Euro-Ficoll prevented edema of the exocrine tissue and resulted in improved separation of islets from the exocrine tissue when compared with the standard Ficoll solution (93).

Hypothermic storage of pancreatic digest in a UW organ preservation solution was found to alter the cell volume and tissue densities of both islets and exocrine tissue resulting in improved separation of the preparation following canine (115) and human islet purification (116). Subsequent experiments identified the low concentration of permeable anions and the presence of the large molecular weight colloid , Hydroxyethyl starch, that prevented cell swelling (117).

A radically different approach to islet purification was attempted following the observation that the osmotic permeability of exocrine tissue is approximately ten times greater than that of islets (118). Preliminary experiments using individual islets and exocrine tissue have shown that a brief 30 second exposure to a hypotonic solution allowed selective lysing of the exocrine tissue without damaging the islets (119).

Due to its simplicity and effectiveness, the density gradient centrifugation method remains the method of choice for purification of islets by many centres. Large-scale purification of islets was introduced by Lake et al. using the Cobe 2991 blood cell processor (120-122). This system was originally designed to separate blood components via centrifugation in a flexible membrane centrifuge. Decreased operating time and the ability to process an entire preparation of tissue digest in a self-contained, sterile disposable tubing set are key advantages of large-scale purification using the Cobe 2991 machine.

Key advances in the techniques used to isolate highly purified islets from human pancreases include the intraductal perfusion of collagenase solution through the main pancreatic duct, the development of a gentle mechanical dissociation technique, followed by purification using the Cobe 2991 cell processor (77).

DONOR VARIABLES

Several investigators have suggested that the condition of the pancreas before excision and the methods used to remove the pancreas can affect the digestion, purification and, ultimately, the recovery of islets (123). Therefore, considerable interest has recently prompted evaluation of multi-organ donor characteristics before pancreas procurement. Gores et al. evaluated the impact of multiple organ and recipient variables on the functional survival of over 300 cadaveric pancreas allografts and reported a correlation between donor hyperglycemia and pancreas allograft survival (124). In donors with recorded blood glucose values > 10.7 mmol/L, the one year graft survival was significantly lower as compared to recipients of pancreas transplants from euglycemic donors.

Analysis of data from a series of 50 human islet isolations by Zeng et al. reported a correlation between donor age, body weight, duration of cold ischemia and the cause of death with the ability to isolate a minimum of 200,000 islets (125). They also found an increased incidence of unsuccessful isolations (< 200,000 islets recovered) from donors that were hyperglycemic. Benhamou et al., from a series of 68 cases using logistic regression analysis, confirmed that donor hyperglycemia was a significant risk factor in the successful recovery of human islets (126).

The age of the donor at the time of procurement has also been identified as a factor that can affect the subsequent recovery of islets. When Zeng et al. grouped isolations from juvenile (2–15 year-old) and old (56–69 year-old) donors together they observed a decreased ability to successfully isolate islets as compared to the islet isolations from adult pancreases (18–55 year-old) (125). This was also observed by Brandhorst et al. from their analysis of 23 human islet isolations (127). A significant increase in islet yields from donors >30 years of age was demonstrated, as compared to donors that were <20 and 20–30 years of age (127). In an attempt to improve the recovery of islets from pancreases from young donors Socci et al. reported that distension of the pancreas via the duct with collagenase solution before the cold storage interval, allowed an increase in islet yield from the 16–29 year-old donor group as compared to the group of pancreases from adult donors (128). Although Ricordi et al. reported large recoveries of purified islets from pediatric donors using a modified automated digestion protocol, this report contained results from six isolations with only one that was <16 years of age (129).

Many of these studies, using relatively small sample sizes and analyzing the data using univariate analysis, have identified body mass index, condition of the pancreatic capsule and collagenase digestion time as variables that influence the success of an isolation procedure (127, 130). These evaluations were useful to indicate trends, but the studies need to be repeated to increase the number of variables examined and a sample size which permits a thorough statistical evaluation.

PANCREAS PROCUREMENT

One factor that is critical to the subsequent recovery and function of islets is procurement of the pancreas (131). It has been stated that the pancreas is the most difficult to procure of all organs procured for transplantation (132). Published reports of human pancreas procurement have been largely technical in nature, describing methods for the combined procurement of the pancreas and the liver (132-138). Procurement of the human pancreas for islet isolation has not been specifically addressed. It has been suggested that edema of the pancreas or degranulation of the exocrine tissue as a result of a suboptimal procurement of the organ can prevent the successful separation of the islets during the purification phase of islet isolation (131).

Rodent studies have suggested that *in situ* vascular flush of the pancreas may result in a decrease in subsequent islet recovery (139-142). Ohzato et al. showed that *in situ* flushing with Hanks' balanced salt solution (HBSS) reduced the recovery of islets as compared to non-flushed pancreases (139). Other studies have confirmed that a rodent pancreas procurement protocol incorporating an *in situ* flush can adversely impact the ability to isolate islets depending on the solution infused (140, 142). Ricordi et al. was unable to recover islets from pig pancreases that had been *in situ* perfused with Euro-Collins solution (143). Hesse et al. reported a 75% reduction in islet recovery following *in situ* vascular flushing with either Collins or silica gel filtered plasma solution as compared to canine pancreases that had not been *in situ* flushed (144). In contrast, both Munn and Zucker et al. have published studies, using the canine model, supporting a brief period of UW perfusion ex vivo

before cold storage (145, 146). Due to species-related differences, this data must be viewed with caution when interpreting it for clinical applications.

WHOLE PANCREAS PRESERVATION

Following procurement, the ability to safely store the pancreas allows sufficient time for transportation of the pancreas to islet processing laboratories, necessary recipient screening and lymphocytotoxic cross-matching (20, 147). Working towards improved preservation methods will facilitate future isolation procedures to be scheduled electively. The majority of published studies on preservation of the pancreas have used the pancreas transplant model. There have been relatively few studies on the preservation of the pancreas before islet isolation. Early attempts to preserve the pancreas focused on those preservation techniques established for kidneys. These techniques appear to have been less successful because of the pancreatic tissue's greater sensitivity to damage during removal and preservation (143, 147). Hyperbaric oxygenation, hypothermic pulsatile perfusion and simple cold storage are three methods used to preserve the pancreas.

Hyperbaric preservation of pancreaticoduodenal grafts was first attempted by Idezuki et al., in 1968, as a means of preserving the functional viability of the pancreas for several hours, while attempting to prevent ischemic damage commonly associated with organ preservation (148). The aim of hyperbaric cold preservation was to inhibit metabolism and prevent tissue edema while maintaining the oxygen concentration at a level that would allow permeation into the tissue. However, the excessively high oxygen partial pressures were harmful to the tissues and irreversible

damage to the pancreas occurred following 24 hours of storage (148, 149). Additionally, technical difficulties associated with this method of preservation made it impractical.

The use of continuous hypothermic perfusion as method to preserve organs began more than 25 years after Belzer described the perfusion device for the preservation of canine kidneys (150). Since that time, a number investigators researching pancreas preservation have used continuous hypothermic perfusion devices before transplantation. The advantages of perfusion systems include the capability of a continuous supply of nutrients (including oxygen), the ability to maintain and control pH and the potential to remove the waste products of metabolism (151). Disadvantages of pancreas perfusion include the complexity of the perfusion device and the requirement that the pancreas be intact and completely vascularized. Additionally, since the pancreas is a low flow organ and requires only a small proportion of the blood flow, problems including edema develop if the pancreas is perfused at too high a pressure or flow rate.

Westbroek et al., using the Belzer kidney perfusion device at a perfusion pressure of 50-60 mmHg, evaluated canine allograft survival following 24 hour hypothermic perfusion using a cryoprecipitated plasma (CPP) solution (152). Allografts survived for approximately 11 days post-transplant. Tersigini et al. found that 24 hour perfusion of canine pancreaticoduodenal grafts in CPP with a human serum albumin and methylprednisolone added to the preservation solution increased allograft survival to approximately 19 days (153). DeGruyl et al. perfused canine pancreases for 24 hours and following allotransplantation found no difference as

compared to simple cold storage (154). Florack et al. also preserved canine segmental allografts for 24, 28 and 72 hours and concluded that cold storage was superior and more reliable than machine perfusion (155).

Another technique, and the most simple alternative, for pancreas preservation is hypothermia alone; simply decreasing the temperature of the pancreas following excision. Following 24 hours of cold storage Toledo-Pereya et al. found no difference in graft survival between Sack's crystalloid solution as compared to a modified silica gel filtered fraction of plasma (156). However, following cold storage of 48 hours, only organs preserved in the silica gel plasma solution resulted in successful transplantations.

Autotransplantation of canine pancreases preserved using simple cold storage in Collins solution for 24 hours was first demonstrated by Baumgartner et al. (157). The importance of these studies is that by autotransplanting the pancreas following storage, longterm survival and function could be evaluated. Florack et al. was successful in extending the duration of storage of the canine pancreas to 48 hours before autotransplantation using a hyperosmolar silica gel filtered (SGF) fraction of plasma solution (158). The longterm survival of 75% of the grafts following 48 hours cold storage in SGF was comparable to the 80% survival observed in the non-preserved control grafts. Extending the preservation interval to 72 hours resulted in only 30% of the grafts surviving longterm.

Wahlberg et al. compared the effects of different cold storage solutions for the preservation of slices of canine pancreas (159). Tissue water was determined and provided a measure of tissue edema that occurred during the preservation interval.

Slices of pancreas stored in Collins solution for a 24 hour period showed a greater degree of edema as compared to slices which were stored in a solution containing lactobionate. Based on these results the University of Wisconsin (UW) organ preservation solution was developed. UW solution contains the large molecular weight impermeants lactobionate and raffinose (to prevent edema), adenosine (as a substrate for energy phosphate production), allopurinol and glutathione (oxygen-free radical scavengers) and hydroxyethyl starch (a colloid to provide oncotic support during flushing) (160, 161). Subsequent experiments using segmental canine transplants following 48 and 72 hours of storage demonstrated the superiority of UW solution (162). This solution has been proven effective for the preservation of canine liver (163), kidney (164), and heart (165) and was eventually incorporated to clinical preservation of the liver (166) and pancreas (167) before transplantation (168). Despite its costs, UW preservation solution has rapidly become the standard *in situ* flush and storage solution for all intrabdominal organs before transplantation.

PANCREAS PRESERVATION BEFORE ISLET ISOLATION

The relationship between the duration of hypothermic cold storage of the pancreas and the ability to recover rodent islets has been demonstrated in several studies (139-142, 169). Kneteman et al. showed a significant decrease in both islet recovery and the ability to secrete insulin *in vitro* following *in situ* vascular flushing and cold storage for 6 hours with a variety of preservation solutions (140). Significantly more islets isolated from cold storage pancreases were required to induce normolgycemia as compared to freshly isolated controls (141). Ohzato et al.

speculated that the pancreatic duct lost integrity with increased cold storage and that distension of the pancreas via the duct at the time of procurement enabled the recovery of islets following the preservation period (139).

Toledo-Pereya et al. reported a decrease in the survival of canine autografts of unpurified pancreatic microfragments isolated following a period of cold storage (170). The group of animals which received transplants of tissue preserved for 48 hours before pancreas dissociation and autotransplantation were successful in 40% of the cases as compared to 60% for animals receiving grafts following 24 hours of storage.

Following *in situ* vascular perfusion with either Collins or silica gel filtered plasma solutions, Hesse et al. reported a significant reduction in islet recovery (144). This reduction was more pronounced with cold storage. In pancreases that had been stored for only 3 hours 40% as many islets were recovered as compared to freshly isolated controls. This decrease progressed to 20% at 6 hours and 12.5% at 24 hours (144).

A significant decrease in islet recoveries was also observed by Munn et al. following vascular flushing and 12 hours of cold storage. Intrasplenic transplantation of the unpurified pancreatic digest was successful in only 1 of 5 animals receiving islets isolated from the stored pancreases as compared to 6 of 7 receiving islets from nonpreserved pancreases (145). Subsequent experiments using pancreatectomy followed by ductal flush of collagenase in UW solution and 24 hours of cold storage showed that both islet recovery and isografts were comparable to the nonpreserved controls. Zucker et al. showed that *in situ* vascular flushing and 24 hour preservation

of canine pancreases followed by islet isolation with purification resulted in 100% autograft success following 24 hours of cold storage (146). Extending the duration of cold storage before islet isolation to 48 hours resulted in 60% of the autografts being successful.

There are limited studies related to clinical storage of the pancreas before islet isolation. It has been shown that storage of the human pancreas in Euro-Collins solution before islet isolation can lead to a significant reduction in islet viability (171). Preliminary investigations using UW solution to store the pancreas before islet isolation suggests that the pancreas can be stored for a period up to 18 hours (141).

ISLET TRANSPLANT REGISTRY

Successful clinical islet transplantation into patients with type 1 insulin-dependent diabetes mellitus has recently been demonstrated by a number of transplant centres worldwide (172-176). As reported to the clinical Islet Transplant Registry, there have been as many as 34 institutions worldwide that have performed clinical adult islet transplants (176-178). From December 12, 1893, through December 31, 1994 a total of 244 human adult islet transplants have been performed. Although the patient one year survival was 95%, islet graft function as assessed using the C-peptide secretion (>1 mg/mL) was 28%. Only 11% of the patients were insulin-independent at one year post-transplant (177, 178). Further analysis by the Islet Transplant Registry of 75 C-peptide negative type 1 diabetic recipients transplanted between 1990 and 1993 identified several criteria that were common

factors to patients being able to withdraw exogenous insulin therapy for greater than 1 week post-transplant (176-178). All insulin-independent patients received islets isolated from pancreases with less than 8 hours of cold storage and received a minimum of 6,000 islet equivalents/kilogram body weight. Islets were transplanted into the portal vein and the induction immunosuppression consisted of antilymphocyte serum (antilymphocyte or antithymocyte globulin) (176-178). When all four of these criteria were met in 24 of the patients, 83% of the patients showed evidence of C-peptide (>1 ng/mL) secretion at one month post-transplant. At one year post-transplant, 29% of these recipients were insulin-independent with 46% having Hb A1c levels below $<7\%$.

One of the remaining obstacles to the establishment of large-scale clinical islet transplantation programs worldwide is the inability to isolate sufficient islet yields required for single donor to single recipient islet transplant. Until such time when sufficient numbers of islets can be isolated from one cadaveric donor pancreas and transplanted into an immunosuppressed longterm diabetic patient, some form of islet storage is needed. Tissue banking through shortterm tissue culture and longterm preservation through cryopreservation are methods currently used to store and accumulate islet tissue (77). Cryopreservation of isolated islets is an effective means to store or bank islets before multiple donor islet transplantation.

CRYOPRESERVATION

A review of the events occurring during the freezing of cells enables one to better understand the processes involved in the cryopreservation of islets. During

cooling, the cell and the surrounding fluid will supercool to about -5°C, depending on the cell type and the solutes present. Continued cooling will result in ice formation extracellularly (179-181). The intracellular water remains unfrozen and in a supercooled state as the cell membrane acts as a barrier to ice crystal growth (179). The presence of ice extracellularly causes concentration of the remaining extracellular solutes to increase and this creates a chemical disequilibrium with the supercooled water intracellularly. Intracellular water will leave the cell in response to this osmotic potential. The rate of water efflux is limited by several factors including the permeability coefficient for water (Lp), cell surface to volume ratio and the vapour pressure of the extracellular and intracellular water (181). As the water leaves the cell it is frozen, thus maintaining the osmotic gradient between the intracellular and extracellular environment (181). If the cooling rate is slow enough, the cell will respond osmotically and shrink thereby decreasing the chance of intracellular ice formation. However, if the cooling rate or the membrane permeability of the cell is such that the rate of efflux from the cell cannot maintain osmotic equilibrium, water trapped within the cell will eventually freeze; causing intracellular ice formation.

The presence of intracellular ice is highly correlated with lethal injuries to the cell membrane which occur primarily during thawing (180, 181). Small ice crystals formed during the cooling phase have a high surface energy and aggregate together during thawing, termed recrystallization. It is the formation of large aggregates of ice intracellularly that is believed to damage the cell membrane. If the cells are

thawing rapidly, the chance of recrystallization occurring is reduced and the cells will survive.

Avoidance of intracellular ice by decreasing the cooling rate is critical to post-cryopreservation survival, however, damage can occur if the cell is cooled too slowly (182). During slow cooling the concentration of the intracellular solutes increase as the cellular water leaves in an attempt to maintain osmotic equilibrium. Slow cooling injury occurs due to the increased intracellular solute concentration. Several hypotheses have been formulated to explain the damaging effects of slow cooling, including: prolonged exposure of the cellular organelles to the high osmolality and hypertonicity of the intracellular solutes lead to protein and lipid destabilization (183, 184); cells have a minimum critical volume beyond which the membrane is distorted and eventually ruptures (185); and, the volume of unfrozen fraction of intracellular solutes is critical and distortion of solute channels result in cell damage (181).

The era of successful cryopreservation was initiated by the accidental discovery of the protective effects of glycerol on the survival of chicken sperm by Polge et al. in 1959 (186, 187). Before this time, scientists had little success recovering viable cells following freezing. The addition of cryoprotectants such as glycerol and later dimethyl sulfoxide (DMSO), to low temperature freezing protocols provided protection during the freeze and thaw process to allow increased recovery of cells following cryopreservation. Cryoprotectants are thought to provide protection by reducing the risk of intracellular ice formation and to prevent against the effects of harmful exposure to high concentrations of intracellular solutes (181). Mazur et al. first showed that slow cooling (0.3°C/minute) of 17 day old fetal rat pancreases

incubated in 2M DMSO allowed 80% survival as assessed by amino acid incorporation of the frozen-thawed pancreases (188). Subsequent experiments by Rajotte and Mazur, found that the age of the fetal pancreas was critical to the post-cryopreservation survival (189). Kemp et al. first demonstrated the ability of cryopreserved fetal pancreases transplanted under the kidney capsule to reverse the experimentally-induced diabetes (190).

The first example of successful cryopreservation of isolated pancreatic islets was demonstrated by Rajotte et al., who, using a protocol similar to that used for fetal pancreases, showed that cryopreserved rodent islets could retain their morphological integrity and physiological capacity to control hyperglycemia following indefinite low temperature preservation in liquid nitrogen at -196°C (191). Islets which were equilibrated with 2 M DMSO and slowly cooled (0.25°C/minute) to -75°C reversed streptozotocin-induced diabetes whereas diabetic rats which received islets cooled at 1.0°C/minute remained diabetic (192). Subsequently, Rajotte et al. showed that if islets were cooled to -40°C before plunging into liquid nitrogen in combination with rapid thawing (200°C/minute) a better *in vitro* functional response was observed as compared to islets which were slow cooled to -75°C (193).

Isolated islets have been successfully cryopreserved using several protocols. A number of these approaches have evaluated different cryoprotectants, alternative methods used to add and remove the cryoprotectant, and have varied cooling and thawing rates (194). Bank et al., using a rapid cooling rate of 75°C/minute in combination with slow thawing (3.5°C/minute) reported islet function during glucose challenge to be comparable to freshly isolated islets (195). A two step cooling

technique was later described by Bank et al. which held cells at an intermediate sub-zero temperature to allow time for the cells to dehydrate before they are plunged into liquid nitrogen for low temperature storage (196).

McKay and Karow showed that islets slowly cooled at 0.3°C/minute to -75°C, had a biphasic insulin release when challenged with a high glucose solution as compared to islets that were cooled at 3.0, 14 and 48°C/minute (197). Andersson and Sandler reported that a faster cooling rate of mouse islets of 5°C/minute to -70°C, survived better than islets cryopreserved at 0.5°C/minute (198, 199). Bretzel et al. used a cryopreservation protocol where rodent islets in 1.3 M DMSO were cryopreserved at 2°C/minute to -35°C followed by cooling at 7°C/minute to -100°C and reported function comparable to freshly isolated islets (200, 201). Finally, in 1989, Rajotte et al. compared a variety of these reported cryopreservation protocols and found that slow cooling (0.25°C/min) to -40°C before plunging into liquid nitrogen in combination with rapid thawing (200°C/minute) resulted in similar *in vitro* function comparable to non-frozen controls (202). Taylor et al. (203, 204) have subsequently confirmed the original finding of Rajotte and showed in cryopreserved rat islet transplantation studies, that slow cooling (0.25°C/minute) to -40°C in combination with rapid thawing (200°C/minute) is superior to slow cooling (0.25°C/minute) to -70°C. Coloumbe et al. confirmed these results in a transplant model where 3000 frozen-thawed rat islets normalized diabetic recipients and showed similar results to animals receiving freshly isolated islets (205). Rich et al. also confirmed that slow cooling to -40°C and rapid thawing was superior to slow cooling to -70°C followed by slow warming at 50°C/minute (206). However, they observed

impaired islet function following transplantation of 750 frozen-thawed islets as compared to non-frozen controls. When the functional losses associated with the cryopreservation process were accounted for, islet function was comparable to non-frozen control isografts.

Control of the cooling and thawing process, as well as the protection of intracellular ice formation, and the large osmotic fluctuations through the addition of a cryoprotectant agent are all critical to the successful recovery of islets following cryopreservation. Using slow cooling to -40°C followed by plunging into liquid nitrogen in combination with rapid thawing, canine islets have been successfully autotransplanted (193, 207, 208) and have been used in allograft studies (209). Porcine islets, both fetal proislets and islets isolated from adult pancreases have been shown to maintain functional viability following cryopreservation (210-212). As well, human islets cryopreserved using the slow cooling and rapid thawing approach retain the ability to respond to a high glucose stimulation *in vitro* (213, 214) and are capable of surviving *in vivo* when transplanted into nude rats (215). Human islets cryopreserved and maintained in a low temperature tissue bank have been used clinically to increase the transplantable islet mass (69, 77, 172, 175).

In addition to being able to safely maintain tissue in a state of preservation with the only potential deleterious effects being background radiation (181), there are many other benefits of cryopreservation. The actual freezing and thawing processes of isolated islets have been shown to extend xenograft survival when frozen-thawed rat islets are transplanted into diabetic mice (216). The expression of Class I MHC in frozen-thawed mouse islets is reduced following cryopreservation and

may be a possible explanation for the prolongation of xenograft survival (217). Using unpurified canine pancreatic microfragments, Evans et al. demonstrated that the processes of cryopreservation act as a purification step by reducing the overall amylase concentration of the tissue while maintaining the insulin content (218). As well, frozen tubes containing cryopreserved islets can be easily transported between collaborative research centres (192, 219). Tissue sterility and functional viability of islets in a low temperature tissue bank can be easily assessed to confirm that only sterile and viable islets are being transplanted.

THESIS AIMS

This thesis addresses two major issues limiting clinical islet transplantation. The first is the lack of consistency and reliability in the ability to isolate human islets of Langerhans and the second is the absence of effective protocols for the large-scale cryopreservation of isolated human islets.

The hypothesis was that the consistency and quality of human islet isolations would be improved by: (1) identifying a superior pancreas procurement protocol, and, (2) development of a more consistent pancreatic islet isolation.

The specific aims of the study were:

I. identification of variables in organ donors that affect the yield and function of islets,

II. determination of limits in the cold storage interval of the pancreas before islet isolation,

III. evaluation of pancreas recovery techniques from cadaveric donors,

IV. development of an automated computer controlled digestion device.

The second issue addresses the lack of effective methods for the cryopreservation of large numbers of human islets. The hypothesis is that the cryopreservation of mass quantities of islets can be optimized. Research projects were completed to develop improved protocols and new techniques to optimize islet cryopreservation.

The specific aims were:

I. investigation of microbial contamination during isolation, *in vitro* culture, and cryopreservation,

II. development of a method for the bulk cryopreservation of islets,

III. evaluation of the methods used for the removal of DMSO following cryopreservation,

IV. development of an automated protocol for the addition and removal of the cryoprotectant.

Inconsistencies in the ability to isolate and purify human islets from donor pancreases suggests that there are pre-existing conditions in certain cadaveric organ donors that affect the ability to isolate viable islets. In an attempt to reduce the variability in yields of human islet isolations and to identify specific donor factors that are potentially deleterious to the recovery and function of isolated human islets, Chapter II retrospectively reviewed 153 human islet isolations performed at our centre over a 3 year period. Factors leading to successful isolations (recovery of >100,000 islet equivalents, at a purity >50%) were retrospectively analyzed using bivariate and multivariate analysis.

One donor factor identified as significantly affecting the ability to isolate human islets of Langerhans was the duration of cold storage before islet isolation. The maximal tolerated duration of preservation of the human pancreas following procurement and cold storage in UW solution before successful digestion and purification is reviewed in Chapter III. A series of 146 consecutive adult human islet

isolations were evaluated in respect to the recovery of islets following collagenase digestion and Ficoll purification and *in vitro* islet function.

The actual methods to procure the pancreas for subsequent islet isolation have been, for the most part, overlooked as a factor that can affect the subsequent islet isolation. Procurement of the human pancreas specifically for islet isolation has not been addressed. As part of the overall goal to improve the methods used to isolate viable human islets, Chapter IV evaluates methods of pancreas procurement before islet isolation comparing *in situ* vascular perfusion with UW organ preservation solution followed by total pancreatectomy to a subtotal pancreatectomy procedure before *in situ* vascular flushing. The experimental parameters to be measured include post-dissociation islet yield, post-Ficoll purification recovery, and the *in vitro* islet function both before and following cryopreservation.

The current methods of isolating and purifying islets are labour-intensive and rely heavily on the operator for successful isolation and purification of large numbers of viable islets. Chapter V describes the development and initial testing of an automated system for the isolation of islets. This system, termed ACES (Automated Cell Extraction System) allows computer control of the islet isolation process. As well, it incorporates a novel method of recombinating dissociated tissue. Preliminary development and testing of the ACES system was performed using the canine pancreas. Following initial design and evaluation to determine optimal system configuration, a series of 12 canine islet isolations were performed.

Limitations in the isolation of large numbers of viable islets from cadaveric donor pancreases make it necessary to collect pools of isolated islets from several

donors to achieve a sufficient transplantable islet mass. Tissue banking through shortterm tissue culture and longterm banking through cryopreservation are the methods used currently to achieve this. The second issue that has limited clinical islet transplant trials to date, is the lack of effective methods for the low temperature banking of human islets. These methods of islet storage have a potential risk of microbiological contamination. Transplantation of microbiologically contaminated islets into immunosuppressed patients has recently been documented (220). In current methods of islet isolation, tissue culture and cryopreservation contain multiple steps where micro-organisms could potentially infect and contaminate the islet preparation. Furthermore, the use of islets from multiple donors can compound the risk of infusion of contaminants into individual recipients. The aim of Chapter VI is to first retrospectively evaluate the frequency of positive microbial culture of isolated islets maintained in a low temperature tissue bank and to secondly retrospectively evaluate the contamination in the steps in the islet isolation, *in vitro* tissue culture and the cryopreservation and storage of isolated human islets.

Current methods of low temperature banking of islets are based on protocols originally developed by Rajotte et al., in 1977, for the cryopreservation of rodent islets (191). These cryopreservation protocols are cumbersome and have an increased risk of microbial contamination due to current practices of placing small aliquots of islets in multiple glass freezer tubes.

The development and application of a commercially available blood freezer bag for the bulk cryopreservation of isolated islets is examined in Chapter VII. Cryopreservation in a freezer bag system will simplify the entire islet cryopreservation

process, minimize tissue loss during recombination, both before and after cryopreservation, and minimize the risk of microbial contamination, all allowing for the maximal recovery of islets.

It is the aim of Chapter VIII to first compare a newly developed protocol, a modified slow step approach, to the standard sucrose dilution and slow step dilution protocols for the removal of the cryoprotectant DMSO following thawing. Known numbers of canine islets were cryopreserved in glass tubes using the standard cryopreservation protocol of slow cooling to -40°C before plunging into liquid nitrogen. Following rapid thawing and removal of the cryoprotectant using the three protocols, islet recovery, *in vitro* and *in vivo* function were compared. The second aim of Chapter VIII is to compare the modified slow step protocol for removal of the DMSO using an automated protocol within the freezer bag system described in Chapter VII.

In Chapter IX, general discussions and conclusions, the results of each study are discussed and compared in relevant findings in the literature.

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II

VARIABLES IN ORGAN DONORS THAT AFFECT THE RECOVERY OF HUMAN ISLETS OF LANGERHANS[1]

INTRODUCTION

Transplantation of isolated islets of Langerhans has been attempted as a treatment for patients with longstanding type 1 diabetes mellitus. Although the overall success is quite low, as judged by insulin-independence post-islet transplant, a significant proportion of these patients have reduced exogenous insulin requirements and have measurable C-peptide secretion for prolonged periods post-islet transplant (1-5). Recent review of past clinical islet transplants by the Islet Transplant Registry identified several critical factors including the transplantation of an islet mass exceeding 6000 IE/kg body weight (5). With the exception of a few cases, this transplantable islet mass is accomplished through the use of multiple donors as the current methods of human islet isolation and purification are inconsistent and rarely recover a sufficient quantity of islets for a 1-donor-to-1-recipient transplant. For islet transplantation to be a viable clinical option methods must be improved. The ability to identify an optimal donor for islet isolation would greatly assist in this goal. The aim of this study was to retrospectively analyze human islet isolation records in an attempt to identify pre-existing donor factors that affect the ability to isolate and purify human islets of Langerhans. The recovery and the

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in vitro function of the isolated islets were also compared to determine if there were any correlations with islet yield and function.

MATERIALS AND METHODS

Cadaveric Donors. Human pancreases were obtained with informed consent from relatives of 175 brain dead cadaveric multi-organ donors from October 1990 to December 1993. Tissue procurement and experimental protocols were approved by the Human Ethics Committee of the University of Alberta Hospitals. A total of 22 pancreases were excluded from our analysis for the following reasons: technical errors during organ procurement resulting in > 10 minutes of warm ischemia (n=2), intra-abdominal organs flushed with Euro-Collins preservation solution (n=5), technical failures in either the digestion or purification phases of the islet isolation (n=8), and isolations with suboptimal collagenase dose or ineffective collagenase lots during pilot testing (n=7). Of the remaining 153 isolations, 67 pancreases were procured by our local procurement team, 43 procured before *in situ* vascular flushing, and the remaining 24 were removed as part of the multi-organ procurement with vascular perfusion using cold University of Wisconsin (UW) organ preservation solution. All locally procured pancreases were processed within 3 hours of recovery. The remaining 86 pancreases were received from distant collaborative transplant centres and were shipped in UW solution on ice after *in situ* vascular flushing with cold UW solution. Requests were made to both local and distant organ procurement teams to avoid obstruction of fluid outflow from the splenic vein during the organ harvest. The duration of cold storage was defined as the time from cessation of

blood flow to the pancreas (cross clamp time during organ harvest) to the time of initial intraductal collagenase distension during the islet isolation process. Additional factors retrospectively reviewed from donor charts included: donor age, sex, BMI (body weight (kg)/height $(m)^2$), time from injury to declaration of brain death, and time from brain death to organ procurement. The minimum and maximum blood glucose as well as the maximum amylase were recorded from the donor charts.

Cause of death was categorized into three experimental groups: 1) traumatic (motor vehicle accidents, motor cycle accidents, and gunshot wounds), 2) non-traumatic (carbon monoxide poisonings, anoxia, tumors, and drug overdoses), and 3) cerebrovascular. Hemodynamic stability of the donor was reviewed including the duration of any hypotensive episodes (minimum systolic blood pressure <90 mmHg or mean arterial pressure <60 mmHg) and the doses of vasopressors administered. The frequency and duration of cardiovascular and respiratory arrests were recorded. Procurement factors, including donor team, procurement location, and surgical method of pancreas removal were all recorded. Subjective assessment of the organs, including the appearance of the gland (fatty and damaged) and the distensibility of the pancreas during intraductal collagenase infusion, was retrospectively recorded from isolation data records.

Islet Isolation. Upon arrival at the islet isolation laboratory, pancreases were cleaned of all vascular, fatty, and connective tissue before the gland was weighed. Two cannulas (16-18 gauge catheters; Medicut, Becton Dickinson, Sandy, UT) were inserted into the main pancreatic ducts and directed towards the head and the tail of the pancreas following dissection of the pancreatic duct in the mid-body of the

gland. An additional 14 gauge Medicut cannula was placed at the head of the gland and all were secured with 3-0 silk.

The pancreases were then placed in a customized perfusion device (6) and perfused under controlled pressure with cold Hanks' balanced salt solution (HBSS; Gibco, Burlington, ON) containing collagenase Type XI and V (Sigma Chemical Co., St. Louis, MO) (n = 121) or collagenase Type P (Boehringer Mannheim, Indianapolis, IN) (n = 32). Four different lots of collagenase were utilized in this study, three from Sigma and one from Boehringer Mannheim. Attempts were made to provide equivalent enzyme concentrations by adjusting the collagenase dose. Pancreases were perfused with cold (4–8°C) collagenase solution while maintaining constant pressure of 80 mmHg for the first 5 minutes and 160–180 mmHg for the next 5 minutes (6). After 10 minutes, the collagenase solution perfusing the pancreas was slowly warmed to 35°C and the distended pancreas was transferred to the continuous digestion device for dissociation (6, 7). Recirculating collagenase solution was held at 37 ± 0.5°C throughout the dissociation phase. Samples of the digest were evaluated during the digestion and when the majority of islets were free from the exocrine tissue the digestion phase was ceased and 8–10 L warm (37°C) HBSS solution supplemented with 2% newborn calf serum (Gibco or Hyclone, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin (P/S) was flushed through the continuous digestion device chamber (8). The effluent was cooled, washed further with cold HBSS, and recombined. Duplicate aliquots of recombined digest were removed and stained with dithizone for pre-purification islet assessment (9, 10). The islet mass was quantified and the purity of the preparation was assessed by two

independent investigators in accordance with the criteria established at the 1989 International Workshop on Islet Assessment (9). The number of islets in each diameter class was determined using an optical graticule. The number of islets was then converted to the standard number of IE (number of islets of 150 μm diameter equal in volume to sample) (9). The total number of IE/gram of processed pancreas was also calculated.

Tissue digest containing endocrine and exocrine tissue was purified using Ficoll (400-DL, Sigma) which had been dissolved in Medium 199 solution (Gibco). Ninety-three of the preparations were purified using discontinuous gradients of Ficoll in 50 mL Falcon tubes. Tissue from 17 preparations were divided with one half bottom-loaded into 50 mL Falcon tubes and purified with discontinuous Ficoll gradients and the other half top-loaded onto continuous gradients in the Cobe 2991 cell separator (COBE Laboratories, Lakewood, CO) (11). The remaining 43 preparations were purified entirely with continuous gradients of Ficoll using the Cobe 2991 machine. Islet recovery following purification was assessed in duplicate by counts of dithizone-stained aliquots of the final suspension of tissue (9). Purity of the preparation was assessed subjectively by comparing the relative quantity of dithizone-stained tissue to unstained exocrine tissue. Islet isolations were considered successful if >100,000 IE at a purity of >50% were recovered following islet purification.

Viability Assessment. After a minimum culture period of 24 hours post-isolation at 37°C in CMRL 1066 tissue culture media (Gibco) supplemented with 10% fetal calf serum (Gibco), 25 mM HEPES (Gibco), and P/S in a humidified atmosphere of 95% air and 5% CO_2, islet function was assessed by perifusion with

glucose (13, 14). Duplicate samples of known numbers of islets were placed in modified Millipore chambers and perfused with Kreb's bicarbonate solution (Gibco) containing 0.5% bovine serum albumin (Sigma) and glucose (Abbott Laboratories, Montreal, PQ) in the following concentrations: initially 2.8 mmol/L for 60 minutes, then 28 mmol/L for 60 minutes, and finally 2.8 mmol/L for the remaining 60 minutes of the perifusion. The effluent was collected at 51, 59, 65, 69, 89, 109, 149, and 179 minutes, and the insulin concentration of perifusate samples was determined with double antibody radioimmunoassay using human insulin standards (15). The stimulation index (SI) and the area under the curve were determined from insulin values during glucose perifusion. The SI represents insulin secretion by the islets during the high glucose (28 mmol/L) stimulation period over the insulin secreted during both periods of low basal insulin secretion pre- and post-stimulation (16, 17). The area under the curve value represents the total stimulated insulin release following high glucose stimulation and is calculated by subtracting the mean basal insulin release from the stimulated values over the 60 minutes of high glucose stimulation (16).

Statistical Analysis. Differences between means were compared using two-tailed t-tests or one-way analysis of variance. Differences between proportions were compared using chi-square tests. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 6.1; SPSS Inc., Chicago, IL) with significance level set at $p<0.05$.

Logistic Regression. Logistic regression analysis was performed to determine the independent effect of various donor characteristics and islet isolation factors. For

models evaluating the donor factors, the following variables were included: sex, donor age, cause of death, BMI, duration of time from acute illness to brain death declaration and to organ procurement, vasopressor support, frequency and duration of cardiac or respiratory arrests, minimum and maximum recorded blood glucose, and the maximum serum amylase. Factors recorded during organ procurement included the procurement team, location, method of organ procurement, and the duration of cold storage before islet isolation. For the model analysis of the islet isolation, the following parameters were included: appearance of the gland, processed pancreas weight, collagenase lot used, distensibility of the gland, collagenase digestion time, purification volume and method (50 mL Falcon tubes, Cobe 2991, or both), post-digestion and post-purification islet recovery (total and recovery per gram of pancreas) and the post-isolation function.

Logistic Regression of Categorized Variables. Forward stepwise logistic regression was used to select statistically significant factors which were then categorized as follows: donor age (2.5–18, 19–28, 29–50, 51–65 years), BMI (<20, 20–25, >25), duration of cardiac arrest (0-5 and >5 minutes), minimum recorded blood glucose (<10, 10–15, >15 mmol/L), duration of cold storage (<3, 3–8, >8 hours), cause of death (non-traumatic, traumatic, cerebral vascular accidents), duration of time from brain death to procurement, and procurement location (local and distant).

Multiple Regression. Stepwise multiple regression was performed to evaluate the donor effects in relation to the post-digestion recovery, post-purification recovery, and functional assessment of the islets.

RESULTS

Overall. The variability in the recovery of human islets of Langerhans from our series of isolations is outlined in Figure II-1. The mean ± SEM post-digestion recovery of islets from all 153 isolations was 274,000 ± 16,600 IE (range 0-1,688,000 IE) or 4057 ± 252 IE/g of processed pancreas. Following Ficoll purification, mean islet recovery was 124,000 ± 8900 IE (range 0–748,000 IE) or 1861 ± 144 IE/g. A total of 54.9% (84/153) of the isolations recovered > 100,000 IE and were considered successful with a mean pre-purification recovery 337,000 ± 23,000 IE from the successful isolations and 195,000 ± 20,000 IE from failed isolations.

In vitro assessment of islet viability following overnight tissue culture at 37°C showed biphasic insulin secretion in successful isolations (Figure II-2). Perifusions from failed isolations showed higher basal insulin secretion and lower first and second phase insulin response to high glucose stimulation as compared with islets collected from successful isolations. The calculated SI for the perifusions were significantly lower in the isolations that failed to recover > 100,000 IE (5.9 ± 0.5 for successful isolations vs. 3.3 ± 0.3 for failed isolations; $p < 0.01$). As well, the area under the curve was lower for failed isolations (20.5 ± 1.5 for successful vs. 12 ± 1.3 failed isolations; $p < 0.01$).

Influence of Donor Variables. Evaluation of the donor characteristics in successful isolations identified several factors that affected the recovery of islets including donor age, BMI, and cause of death (Table II-1). Uncontrolled hyperglycemia with recorded blood glucose values > 10 mmol/L in the donors was associated with reduced isolation success. In unsuccessful isolations, the minimum

recorded blood glucose was significantly higher than those of successful isolations (11.2±0.5 mmol/L vs. 7.3±0.2 mmol/L; $p<0.01$). Maximum recorded amylase was significantly higher in failed isolations ($p=0.02$) compared with successful isolations (Table II-1). Eighty-seven percent (n=133) of the donors required either Dopamine or Levophed with 35.4% (n=47) of these requiring elevated doses. In donors requiring high levels of vasopressors (>15 μg/kg/min Dopamine or >5 μg/kg/min Levophed) to maintain blood pressure only 19% (n=9) of the 47 isolations yielded >100,000 IE ($p<0.01$). Other donor factors found to be significant included the maximum Dopamine dose, and the frequency and duration of cardiac and respiratory arrests (Table II-1).

Donor characteristics that failed to demonstrate significant differences between successful and failed isolations included donor sex ($p=0.06$) and ABO blood type ($p=0.38$). The duration of time from onset of acute illness to organ procurement was found not to significantly affect the success rate of the isolations (73.3±9.5 hours vs. 95±15.8 hours, $p=0.29$). While the time from the declaration of brain death to organ procurement was significantly shorter in successful isolations (13±0.5 hours vs. 15±0.5 hours, $p<0.05$) the clinical importance of such an observation is questionable.

Organ Procurement. Pancreases procured locally had a significantly higher success rate as compared with organs procured distantly (Table II-2). The duration of cold storage before islet isolation ranged from 30 minutes to 26 hours with the mean cold storage time from successful isolations being significantly shorter than from failed isolations (5.0±0.5 hours vs. 8.8±1.1 hours respectively, $p<0.01$). The

subjective appearance of the pancreases as excessively fatty ($n = 11$) or damaged upon arrival ($n = 14$) was associated with a reduced isolation success ($p < 0.01$).

Islet Isolation Variables. The collagenase digestion time was significantly shorter in the successful isolations as compared with those that were considered failures (25.9 ± 0.7 minutes for successful isolations as compared with 30 ± 0.9 minutes for failed isolations). Isolation factors which failed to show significance included processed pancreas weight, distensibility of the pancreas, and the purification method. Additionally, using bivariate analysis we found no difference between the four lots of collagenase used in our study.

There was a significantly increased proportion of islets with a mean diameter < 100 μm in the failed isolation group as compared with successful isolations (39.9 ± 2.2 vs. 30 ± 1.4, respectively, $p < 0.01$). This could be indicative of fragmentation of the islets as a result of suboptimal isolation conditions, or pancreas condition.

Logistic Regression Analysis of Predictive Factors for Success in Human Islet Isolations. Stepwise logistic regression analysis of donor and related factors for predicting the success or failure of human islet isolations identified increasing donor age (OR 1.1), higher BMI (OR 1.3), and the local procurement team (OR 7.04) as positively correlated factors and increasing minimum recorded blood glucose (OR 0.68), the duration of cardiac arrest (OR 0.81) and the duration of cold storage before islet isolation (OR 0.86) as negatively correlated factors (Table II-3). This model analysis had an overall prediction accuracy of 82% with a 77% accuracy when predicting a successful isolation and 86% accuracy when predicting a failed isolation.

Logistic regression analysis of the isolation variables identified the increased collagenase digestion time (OR 0.88) and the proportion of islets <100 μm in diameter (OR 0.94) as being predictive factors of isolation failures with a model accuracy of 87%.

Analysis of Categorized Variables. Factors initially identified following logistic regression analysis were categorized and re-analyzed. The proportion of isolations that were successful by category is shown in Table II-4. We observed an increase in isolation success rate with increasing age. However, when these results were compared with the *in vitro* function of the islets isolated, a significant decrease in the insulin secretory capabilities of the islets isolated from the 50–65 year-old age group was observed as compared with the 2.5–18 year-old group ($p<0.02$; Table II-4).

Elevated blood glucose during hospitalization correlated with reduced isolation success. Only 1 of 16 (6%) isolations from the group of donors with persistent hyperglycemia (all recorded blood glucose values exceeding 15 mmol/L) were successful as compared with donors with recorded blood glucose values <10 mmol which were 69% successful ($p<0.01$).

Isolations from the group of donors with a BMI >25 were successful in 73% of the isolations as compared with the group of donors with a BMI <20 which were successful in 36% of the isolations ($p<0.01$).

Logistic Analysis of Categorized Variables. Logistic regression analysis of categorized donor variables identified donors >29 years old as being significant factors affecting isolation success (Table II-5). The groups of donors with: 1) an

elevated minimum blood glucose >15 mmol/L, 2) a BMI of >25, 3) a cardiac arrest >5 minutes in duration, and 4) a duration of cold storage before islet isolation >8 hours were all significant variables when categorized. This model prediction accuracy was 76% for successful isolations and 89% for failed isolations, for an overall accuracy of 84%.

Multiple Regression Analysis. Forward stepwise multiple regression analysis with islet recovery per gram of processed pancreas as the outcome variable identified BMI ($\beta = 0.17$), duration of cardiac arrest ($\beta = -0.31$), elevated vasopressors ($\beta = -0.2$) and local procurement team ($\beta = 0.23$) as donor factors affecting isolation success. Processed pancreas weight ($\beta = -0.28$) and collagenase digestion time ($\beta = -0.25$) were identified as predictive factors in the islet isolation process (Table II-6A).

Multiple regression analysis of the donor and isolation variables' effect on the post-isolation *in vitro* function of the isolated islets identified the local procurement team ($\beta = 0.37$) and minimum recorded blood glucose ($\beta = -0.25$) and duration of cold storage ($\beta = -0.34$) as significant factors (Table II-6B).

DISCUSSION

Although the feasibility of clinical islet transplantation has been successfully demonstrated in several centres worldwide, the overall success rate as judged by insulin independence remains low. Recent analysis by the Islet Transplant Registry of 55 reported adult islet allografts in C-peptide negative type 1 diabetic recipients demonstrated insulin independence at 1 year post-transplant in 11% the recipients (5). Basal C-peptide >1 ng/mL was observed in 33% of the patients (5).

Additionally, the review identified several factors critical to post-transplant insulin independence. These included: 1) transplantation of >6000 IE/kg body weight, 2) intraportal transplantation site, 3) mean cold storage of the pancreas before processing of <8 hours, and 4) induction immunosuppression using ALG/ATG/ALS or OKT3 (5). When all four criteria were met, the post-transplant insulin independence rate at 1 year follow-up increased to 27%. Still, the number of patients that became and remained insulin independent following islet transplantation is low. Standardization of pre-transplant assessment of the islets, transplantation of sufficient numbers of islets, and continued trials in many centres worldwide should improve post-transplant success.

Many factors limit the ability to isolate and purify sufficient numbers of islets for transplantation. Our experience over several years of isolating human islets suggests that there are many factors that affect the ability to recover human islets. The study aims were to retrospectively evaluate 153 consecutive human islet isolations and identify variables in both the multi-organ cadaveric donor and in the islet isolation process to account for the wide variation in recovery following islet isolation.

One factor that was found to be significant in both bivariate and multivariate analysis was age of the donor. Effective separation of the islets from the exocrine tissue is critical to the recovery of islets following purification and islets from pediatric donors were difficult to completely separate from the exocrine components without causing excessive fragmentation. This may relate to the islet ontogeny and collagen composition in the juvenile pancreas. We found a significant decrease in

isolation success in donors < 18 years of age. While our findings are in contrast to a previous evaluation of donor variables by Benhamou et al., they concur with other published experience (20). When Zeng et al. examined isolation success by age, they found isolations from young (2–15 year-old) donors and old (56–69 year-old) donors when grouped together were associated with reduced isolation success as compared with young adult pancreases (18–55 year-old) (21). In their evaluation of donor data and organ procurement on the isolation of human islets, Brandhorst et al. demonstrated a significant increase in islet yields from donors >30 years of age as compared with donors that were <20 and 20–30 years of age (22).

Socci et al. reported that injection of collagenase immediately following procurement, before the cold storage interval, allowed an increase in islet yield from 16–29 year-old donor groups as compared with adult donors (23). Although Ricordi et al. reported large recoveries of purified islets from pediatric donors using a modified automated digestion protocol, this report contained results from six isolations with only one that was < 16 years of age (24). An isolation protocol based on the age of the donor should be considered to improve the recovery of isolated islets.

When age of the donor was categorized, post-purification yields increased with increased donor age; the group of isolations from donors >50 years of age yielded >100,000 IE in 83% of the isolations. Post-isolation islet function as measured using the glucose perifusion assay was significantly reduced in the donors >50 years of age as compared with the 2.5–18 year-old group. Our observations support findings by Sodoyez et al. who reported a decrease in insulin, pro-insulin, and islet-amyloid

polypeptide content from isolated human islets from donor pancreases >50 years of age (25). It was not determined if the reduced beta cell reserve was a result of decreased hormone content or of a reduced number of beta cells within the islets (25). It is important to note, however, that our data reveal no difference in *in vitro* stimulated insulin output from donors >50 years when compared with donors 19–28 or 29–50 years of age. As such, our data supports the continued use of donors >50 years of age for islet isolation and transplant.

The relationship between donor hyperglycemia and pancreas allograft survival was first reported by Gores et al. who found the 1 year graft survival significantly lower (48% vs. 71%) in recipients who received a pancreas transplant from a donor with recorded blood glucose values >200 mg/dL (26, 27). They suggested that the poor graft survival may be a reflection of the decreased beta cell mass which may lead to decreased ability to weather acute rejection episodes as compared with recipients receiving organs from normoglycemic donors (26). Additionally, it was hypothesized that hyperglycemic donors with a decreased beta cell reserve would be more susceptible to specific detrimental cytokines which may be up-regulated during the physiological instability occurring during the time of brain death (27).

The observation of hyperglycemia and poor islet isolation outcome has been reported and confirmed in other donor variable studies including our present evaluation (20, 21). In attempting to account for the poor isolation success, it has been suggested that during the post-stroke death, hyperglycemia may be a result of perturbed hormonal control (catecholamines and cortisol), an endogenous stress response or a result of the glucose based intravenous solutions used during

resuscitation (26). Hyperglycemia in the donor is a factor that leads to decreased isolation success (20, 26). Our evaluation examined both the minimum and maximum serum blood glucose recorded in our donors. Both the minimum and maximum observed blood glucose levels were significant in bivariate analysis, however, only the minimum observed blood glucose was significant following multivariate logistic and regression analysis.

In univariate, logistic and multiple regression analysis we observed that donors increased BMI resulted in a significantly higher recovery and an increased likelihood of isolation success. Although it is not known if these donors have more islets, Wrenshall et al. observed that the pancreatic insulin content was positively correlated with increased body surface area (28).

Regarding the methods of organ procurement and duration of cold storage of the pancreas, our evaluation found no significant influence of the method of pancreas removal on isolation success; supporting a randomized comparative study that found no difference in islet yield or function from pancreases removed before or after *in situ* vascular flushing with UW preservation solution (16).

The duration of cold storage before islet isolation has been previously identified as a significant factor affecting islet recovery (5, 8, 29, 30). Our previous evaluation of the effect of cold storage on the ability to isolate islets from adult (> 18 years of age) pancreases determined that periods of cold ischemia > 16 hours resulted in a significant decrease in post-digestion and post-purification recovery of islets (29). The *in vitro* viability of islets isolated from pancreases with prolonged cold storage (> 16 hours) was also markedly reduced which led us to conclude that

with the current available methods of organ procurement, organ storage and methods used to isolate islets, it is not feasible to process pancreases with >16 hours of cold ischemia (29). The present retrospective analysis of factors including the donor related factors, procurement method, and factors in the isolation, has confirmed that the duration of cold storage following organ procurement is a significant factor which affects the ability to recover large numbers of viable islets.

Previous evaluations have demonstrated that the lot and type of collagenase enzyme can significantly affect isolation success (20). Pancreases processed in our study were digested using four different lots of collagenase. Bivariate analysis showed no difference between the lots of collagenase. A recently developed new collagenase with purified enzyme blends appears to be a more effective and specific method of freeing the islets from the exocrine tissue while sparing the islets from enzymatic damage (31). This new blend may contribute to the consistency and reproducibility of islet isolations.

The models produced by logistic and multiple regression analysis have practical applications for predicting islet isolation success and the number or *in vitro* function of islets that will be isolated. When a donor pancreas is being offered for islet processing, the values for each of the significant variables from our models are obtained and multipled by the logistic regression model's R values to calculate a number (Table II-3). The closer this number is to 1, the greater the likelihood of a successful isolation yielding >100,000 islets at a purity exceeding 50%. Using the multiple regression analysis model the number of islet equivalents per gram of processed pancreas can be estimated by multiplying the value for each significant

variable by the calculated B coefficient (Table II-6A). The *in vitro* function of the isolated islets can be estimated by inserting the donor factors into the model (Table II-6B).

CONCLUSION

This study confirms some recent observations and identifies new factors that can affect the ability to recover large numbers of viable islets. Several donor, pancreas procurement and islet isolation process factors were found to be statistically significant using bivariate analysis. Multivariate logistic regression analysis identified donor age, local procurement team and BMI as factors which were positively correlated with isolation success. Elevated minimal blood glucose, frequency and increased duration of cardiac arrests, and a prolonged duration of cold storage before islet isolation were identified as factors which were negatively correlated with isolation success. Many of these variables were confirmed using multiple regression analysis.

The decision to process pancreases following review of pertinent donor factors should improve the consistency of human islet isolations supporting continued trials in the transplantation of isolated islets in type 1 insulin-dependent diabetic recipients.

TABLE II-1:

Influence of donor characteristics on the recovery of islets isolated from human pancreases.

Variable	Successful Isolations (n=84)	Failed Isolations (n=69)	p Value
Age (years)	40.9±1.4[a]	29.1±1.7	<0.01
Body mass index (kg/m^2)	25±0.45	22.6±0.44	<0.01
Cause of death			
Traumatic	47 (70%)[b]	20 (30%)	<0.01
Cerebrovascular	33 (50%)	33 (50%)	
Non-traumatic	4 (20%)	16 (80%)	
Serum glucose (mmol/L)			
Minimum recorded	7.3±0.2	11.2±0.5	<0.01
Maximum recorded	11.1±0.5	14.9±0.9	
Maximum amylase (IU/L)	85±10	131±18	=0.02
Maximum dopamine dose (μg/kg/min)	7.9±0.6	10.8±0.8	=0.03
Elevated vasopressors			
Yes	9 (19%)	38 (81%)	<0.01
No	76 (68.5%)	35 (31.5%)	
Arrests			
Yes	21 (32.8%)	43 (67.2%)	<0.01
None	63 (70.8%)	26 (29.2%)	
Cardiac	2 (8%)	24 (92%)	
Respiratory	19 (50%)	19 (50%)	

[a] mean±SEM

[b] frequency (percentage of total)

TABLE II-2:

Influence of organ procurement variables on the ability to recover islets.

Variable	Successful Isolations	Failed Isolations	p Value
Procurement team			
Local	46 (68.7%)[a]	21 (31.3%)	=0.03
Distant	38 (44.2%)	48 (55.8%)	
Local procurement technique			
UW flush	20 (83.3%)	4 (16.7%)	=0.053
Primary	26 (60.5%)	17 (39.5%)	
Pancreatectomy			
Duration of cold storage (hours)	5.0±0.5[b]	8.8±1.1	<0.01
Pancreas appearance			
Acceptable	77 (60%)	51 (39%)	<0.01
Fatty infiltrates	4 (36%)	7 (63%)	
Damaged	3 (21.4%)	11 (78%)	

[a] frequency (percentage of total)

[b] mean±SEM

TABLE II-3:

Stepwise multivariate logistic regression analysis of 19 donor related variables predicting isolation success (>100,000 IE, at a purity >50%).

Variable	p Value	R Value	Odds Ratio[a]
Donor age	<0.05	0.18	1.1
Body mass index	<0.01	0.19	1.3
Minimum blood glucose	<0.01	-0.24	0.68
Duration of cardiac arrest	<0.01	-0.17	0.81
Procurement team Local/Distant	<0.01	0.21	7.04
Duration of cold storage	<0.05	-0.13	0.86

[a] Odds ratio = >1 positive correlation with isolation success, <1 negative correlation

TABLE II-4:

Relationship between donor age categories and *in vitro* islet function using the calculated stimulation index.

Donor Age Category	n	Isolation Success Rate[a]	Calculated Stimulation Index[b]
2.5–18 years	23	13%	6.3 ± 1.2
19–28 years	36	37%	5.0 ± 0.9
29–50 years	70	65%	5.2 ± 0.5
51–65 years	24	83%	4.5 ± 0.5[c]

[a] Successful isolation = > 100,000 IE at purity > 50%, $p < 0.05$

[b] mean ± SEM

[c] $p < 0.05$ from the 2.5–18 year-old group

TABLE II-5:

Stepwise multivariate logistic regression analysis of categorized donor variables.

Variable	p value	R value	Odds Ratio
Donor age categories			
(2.5–18 vs. 19–28 years)	=0.256	0.00	2.98
(2.5–18 vs. 29-50 years)	<0.01	0.16	10.5
(2.5-18 vs. >50 years)	<0.01	0.24	81.6
Minimum blood glucose			
(<10 vs. 10–15)	=0.177	0.00	8.3
(<10 vs. >15)	=0.011	0.15	46.4
Duration of cardiac arrest			
(<5 vs. >5 mins.)	<0.01	0.29	19.9
Body mass index			
(<20 vs. 20–25)	=0.06	0.09	4.6
(<20 vs. >25)	<0.01	0.17	11.9
Duration of cold storage			
(<3 vs. 3–8 hours)	=0.79	0.00	0.85
(<3 vs. >8 hours)	<0.01	0.19	7.46

TABLE II-6:

Multiple stepwise regression analysis of donor and islet isolation factors.

A) Post-purification islet recovery (IE/g pancreas) as outcome variable.

Variable	B Coefficient	β Value	p Value
Donor factors			
Body mass index	61.7	0.17	=0.03
Duration of cardiac arrest	-119	-0.31	<0.01
Local procurement	612	0.23	<0.01
Elevated vasopressors	-88	-0.2	<0.01
Islet isolation factors			
Pancreas weight	-17.8	-0.28	<0.01
Collagenase digestion time	-49	-0.25	<0.01

B) Stimulation index calculated from glucose perifusion as outcome variable.

Variable	B Coefficient	β Value	p Value
Donor factors			
Minimum blood glucose	-0.96	-0.25	<0.01
Local procurement	3.0	0.37	<0.01
Duration of cold storage	-0.19	-0.34	<0.01

Figure II-1:

Post-digestion (solid box) and post-purification (grey box) recoveries of islets from 153 human isolations performed over a 3 year period using an identical isolation protocol.

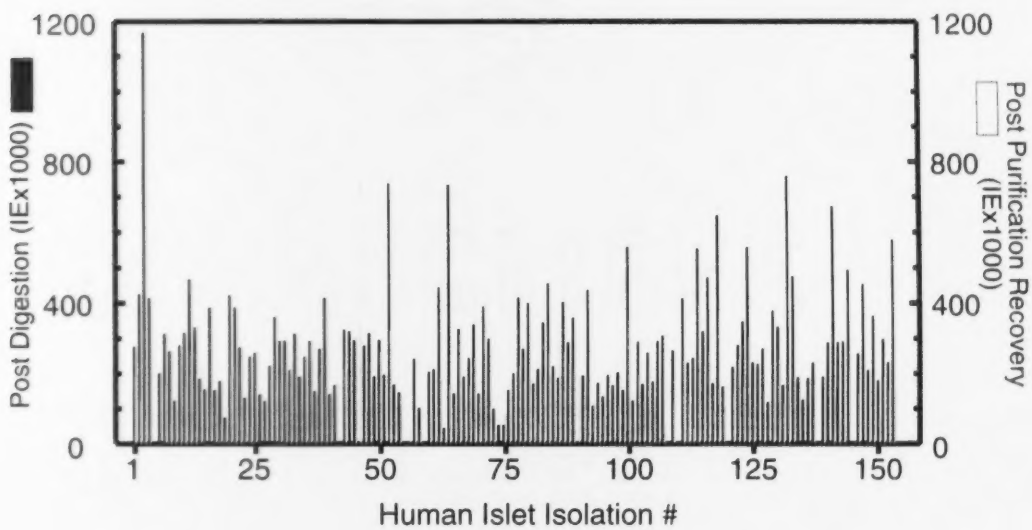
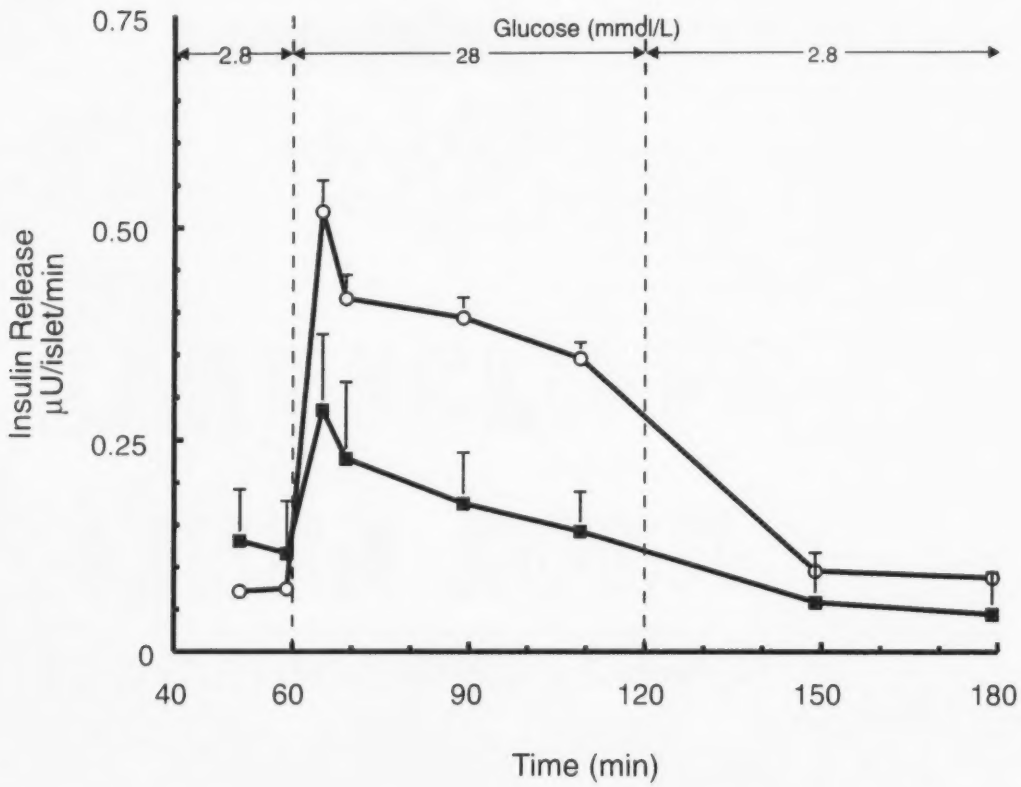


Figure II-2:

In vitro function of isolated islets using glucose perifusion system (mean±SEM). Islets isolated from successful isolations (open circles) vs. islets from isolations yielding <100,000 IE (closed squares).



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III

HUMAN PANCREAS PRESERVATION BEFORE ISLET ISOLATION: COLD ISCHEMIC TOLERANCE[1]

INTRODUCTION

Islet transplantation may become a viable treatment option for patients with insulin-dependent diabetes mellitus (IDDM). To achieve this goal, transplantation of an islet mass exceeding 6,000 islet equivalents (IE) per kilogram recipient body weight must be carried out (1). In selected cases, transplantation of this critical mass has allowed for insulin independence post-transplant (1-4). One of the remaining obstacles to widespread application of clinical islet transplantation has been the inconsistency of islet yields required for successful transplantation. Current methods used to isolate and purify human islets have not advanced to the point where sufficient islets can be reliably recovered from one cadaveric donor for transplantation into an immunosuppressed patient with long standing IDDM. At present, there is a frequent need for pooling organs and combining islet preparations to achieve the required islet mass (1, 4). To do this effectively, a need exists for the cooperation of donor centres to ship pancreases to specialized islet isolation centres that can effectively separate and purify islets. Consequently, the duration of cold storage that can be tolerated by the donor pancreas while in transit before islet processing becomes of critical importance. The University of Wisconsin (UW) organ preservation solution has been proven to be effective in short term pancreas

[1] A version of this chapter has been published. Lakey JRT, Rajotte RV, Warnock GL, Kneteman NM. 1995 Transplantation. 59: 689.

preservation leading to successful vascularized organ transplantation in experimental animal models and clinically (7-9). At present, data are lacking with respect to the maximal tolerated duration of cold storage of the pancreas in UW solution before successful digestion and islet purification. It was the aim of this study to retrospectively evaluate islet isolation records and determine the interval of cold storage where large numbers of viable islets could be successfully cleaved from pancreatic tissue and purified.

MATERIALS AND METHODS

We have retrospectively evaluated islet isolation records collected from 230 consecutive pancreases processed at the islet isolation laboratory during the past 5 years (July 1988 to January 1994). Pancreases were recovered from brain dead cadaveric donors following consent. A total of 33 pancreases recovered were not included in our evaluation for the following reasons: 16 were excluded from donors who were <17 years old; 10 were excluded due to technical failures in either the digestion or purification phases of the isolation; 5 were excluded because the pancreas had been flushed with Euro-Collins preservation solution; and, 2 were excluded because of excessive warm ischemia during pancreas procurement. Of the remaining 197 pancreases, 51 of the islet isolations were performed using the manual trituration method and were removed from our evaluation. From the remaining 146 isolations, 56 pancreases were procured by our local organ procurement team; 33 of these were procured before *in situ* vascular flush and loaded with collagenase solution using previously described techniques (local load group) (10). Briefly the pancreas

was immersed in saline slush (4°C), following procurement the main pancreatic duct cannulated, and collagenase solution was injected by hand over 1-2 minutes into the gland. The remaining 23 locally procured pancreases were removed following *in situ* vascular perfusion using UW solution as part of a multi-organ procurement (local UW flush group). All pancreases from these control groups were processed within 3 hours of recovery. The remaining 90 pancreases were received from distant collaborative transplant centres and were shipped in UW solution on ice after *in situ* vascular flushing with UW solution (distant flush groups). Harvesting teams were requested to avoid obstruction of outflow from the splenic vein during procurement. Pancreases were placed into experimental groups based on the duration of cold storage (3-26 hours) before islet isolation.

Islet Isolation. Pancreases were stored in wet ice (4°C) and shipped to the islet isolation laboratory for processing. Islets were isolated using previously described techniques of collagenase digestion and Ficoll purification (8, 9). All vascular, fatty, and connective tissue was removed and the gland was weighed. Two cannulas (Medicut 16-18 gauge catheter) were inserted into the main pancreatic duct and directed towards the head and tail of the pancreas following dissection of the pancreatic duct in the mid-body of the pancreas. If the main pancreatic duct was not cannulated at the head of the gland during the initial procurement, a 14 gauge Medicut catheter was inserted and secured with 3-0 silk ties.

The pancreas was then placed in a customized perfusion apparatus (Dr. R.V. Rajotte, University of Alberta) and perfused with cold (4°C) Hanks' balanced salt solution (HBSS; Gibco, Burlington, ON) containing collagenase (Type XI and Type

V; Sigma Chemical Co., St Louis, MO). Pancreases were perfused with collagenase solution while maintaining constant pressure of 60-80 mmHg for the first 5 minutes and 160-180 mmHg for the next 5 minutes. After 10 minutes, collagenase solution perfusing the pancreas was slowly warmed to 35°C and then transferred to the continuous digestion device (CDD) for dissociation (8). Recirculating collagenase solution was held at 37±0.5°C throughout dissociation of the pancreas. Samples were evaluated during the digestion and when the majority of islets were free from exocrine tissue the digestion phase was ceased and warm (37°C) HBSS supplemented with 10% newborn calf serum (Hyclone, Logan, UT) and penicillin/streptomycin was flushed through the CDD chamber. The effluent was cooled, washed further with HBSS, and recombined. Duplicate aliquots of recombined digest were removed and stained with dithizone for pre-purification islet assessment (11).

Tissue digest containing endocrine and exocrine tissue was purified using discontinuous gradients of Ficoll (400-DL, Sigma). The last 44 isolations have been purified with continuous gradients of Ficoll using the Cobe 2991 machine (Cobe-BCT Inc., Lakewood, CO). Islet recovery following purification was assessed in duplicate by counts of dithizone-stained aliquots of the final suspension of tissue. The islet mass was quantified and the purity of the preparation was assessed by two independent investigators in accordance to the criteria established at the 1989 International Workshop on Islet Assessment (11). The crude number of islets in each diameter class was determined using an optical graticule. The crude number of islets was then converted to the standard number of IE (number of islets of 150 μm diameter equal in volume to sample). Purity of the preparation was assessed

subjectively by comparing the relative quantity of dithizone-stained tissue to unstained exocrine tissue. Islet yields per gram of processed pancreas were calculated in IE. Islet isolations were considered successful if more than 100,000 IE at a purity of >50% were recovered following islet purification.

Viability Assessment. After a minimum culture period of 24 hours at 37°C in CMRL tissue culture media (Gibco) supplemented with 10% fetal calf serum (Gibco), and penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO_2, islet function was assessed by glucose stimulated perifusion (12, 13). Duplicate samples of known numbers of islets were placed in modified Millipore chambers and perfused with Krebs (Gibco) solution containing glucose in the following concentrations: initially 50 mg/dL for 60 minutes, then 500 mg/dL for 60 minutes, and finally 50 mg/dL for the remaining 60 minutes of the perifusion. The effluent was collected at 51, 59, 65, 69, 89, 109, 149, and 179 minutes, and the insulin concentration of perifusate samples was determined using the double antibody radioimmunoassay (14). The stimulation index (SI) was determined from insulin values during glucose perifusion and represents insulin secretion by the islets during the high glucose (500 mg/dL) stimulation period over the insulin secreted during both periods of low basal insulin secretion pre- and post-stimulation (15). Islets were considered viable if they had a SI of >2.

Statistical Analysis. Islet counts following digestion and islet recovery following purification were compared between control non-flushed pancreases and pancreases that had been subjected to *in situ* vascular flushing and various durations of cold storage before islet isolation. The pancreases were divided into experimental groups

of 3-8, 8-16, and >16 hours based on the duration of cold storage before islet isolation. All results are expressed as mean±SEM. Statistical analysis was performed using the SPSS statistical software package using first ANOVA and then Student's t-tests between the individual groups. Differences between the experimental groups were considered significant when $p<0.05$.

RESULTS

Eighty-eight percent of the isolations from pancreases locally procured and removed before *in situ* flushing (local load) were successful in yielding >100,000 IE at purity >50% (Table III-1). Recovery of >100,000 IE at a purity of >50% in the local UW flush group was 83%, 86% in the UW 3-8 hour storage group, and 73% in the UW 8-16 hours storage group (all p=ns). Pancreases stored for more than 16 hours before islet isolation were successful in only 38% of the isolations ($p<0.05$ vs. local UW flush group).

The recovery of islets following collagenase digestion from locally procured pancreases was 5118±586 IE/g (mean±SEM) and 4544±412 for the local UW flush group (p=ns) (Figure III-1). For pancreases that were cold stored for 3-8 hours 3440±324 IE/g were recovered (p=0.014 vs. local UW flush group) and 4162±548 IE/g after 8-16 hours of cold storage. Storage for >16 hours before isolation resulted in a substantial decrease in islets recovered (2085±607 IE/g, $p<0.001$ vs. local UW flush group).

Islet recovery after Ficoll purification is illustrated in Figure III-2. There were equal recoveries of purified islets from the locally procured groups (3035±271 IE/g

of pancreas recovered from the local load groups vs. 3017±428 for the local UW flush group) (p=ns). As the duration of cold storage before islet isolation increased, the mean recovery of islets decreased (1955±210 for 3-8 hour group and 1745±273 for the 8-16 hour group) with a dramatic decrease observed from pancreases with >16 hours of cold storage (947±333) ($p<0.05$ vs. local UW flush group). As well, there were significantly lower recoveries of islets between the 8-16 hour and the >16 hour storage groups ($p<0.002$).

The viability of the preparations as assessed by *in vitro* glucose perifusion is illustrated in Figure III-3 which demonstrates functional viable islets with dynamic biphasic insulin secretion from islets isolated from pancreases with up to 16 hours of cold storage. The insulin secretion in the perifusion graph of the islets from pancreases with >16 hours of cold storage appeared flat and relatively unresponsive to the high glucose stimulation as compared to the response of islets from the other groups. Insulin release was significantly better from recovered pancreases stored for <8 hours (local load, local UW flushed, or distant recovery and storage <8 hours) when compared to pancreases stored for any period >8 hours ($p<0.01$).

An alternate method to assess the viability response of islets is the calculated SI (Figure III-4). There was no difference in the SI from the local controls (6.9±.73 for local load vs. 6.8±.95 for local UW flush, p=ns). Pancreases with any duration of cold storage >3 hours before islet isolation had a significant decrease in function (4.3±.43 for 3-8 hour group, 3.5±.37 for 8-16 hour group, and 1.6±.25 for the >16 hour group; $p<.023$ for 3-8 hour, $p<.018$ for 8-16 hour and $p<.001$ for > 16 hour

group). A second statistically significant decrease was observed between the 8-16 hour group and the >16 hour group ($p < 0.002$).

DISCUSSION

Several studies have demonstrated a strong correlation between the ability to recover viable islets and the duration of hypothermic storage of the pancreas with various preservation solutions. Animal studies have demonstrated that viable islets can be isolated from pancreases that have been hypothermically preserved for up to 24 hours (16). In the studies of Kneteman et al. (16), following 6 hours of cold storage, the highest number of islets recovered were from rat pancreases that had undergone ductal distension with UW solution without vascular flushing. Extending the cold storage interval to 24 hours resulted in islets being isolated in substantial numbers only from the ductal distension group. Additionally, when cold stored pancreases were utilized, more islets were required to induce normoglycemia than when non-stored pancreases were used (17). Ohzato reported that the pancreatic duct in the rat rapidly lost integrity with cold storage resulting in poor distension with as little as 6 hours preservation time (18). He proposed that distension of the pancreatic duct with preservation solution containing collagenase at the time of organ harvest may allow for better recovery of islets after pancreas storage. Subsequent studies have revealed significant problems with this approach because components of the UW solution resulted in an inhibition of collagenase (19). No islets were recovered by Ohzato when the rat pancreas was distended with UW or Sacks solution containing collagenase, even after 6 hours of cold storage (18).

Hesse reported in canine studies a 75% reduction in islet recovery following *in situ* flushing with either Collins or silica gel filtered plasma solutions in comparison to pancreases from dogs that had not been *in situ* flushed (20). The reduction was more pronounced with cold storage. In pancreases that had been stored for 3 hours only 40% as many islets were recovered, the decrease progressed to 20% recovery at 6 hours and 12.5% after 24 hours (20). Munn and Zucker have subsequently published studies supporting a brief period of UW perfusion ex vivo before cold storage (21) and primary total pancreatectomy followed by low volume ex vivo vascular flush with UW or silica gel filtered plasma before cold storage (22). Our own prospective randomized trial of human pancreases recovery technique revealed no significant impact on islet recovery or *in vitro* function when primary pancreatectomy or *in situ* flush with UW solution were performed (10). Against this background of somewhat conflicting studies, we have evaluated the impact of increasing cold storage on human pancreases before islet isolation.

A significant decrease was observed in the pre-purification islet yield from pancreases stored for 3-8 hours before islet isolation as compared to the local UW flush group. The decrease may be a statistical abberation since the post-purification recovery of islets returned to the levels expected as compared to the trend of decreased islet recovery with increased duration of cold storage observed in the 8-16 and >16 hour groups.

Our retrospective analysis of donor records from 146 human islet isolations indicate that there is a significant decrease in the post-digestion recovery of islets after more than 16 hours of cold storage before islet isolation. Warnock et al. noted

a decrease in islet function from human pancreases that had prolonged storage in Euro-Collins preservation solution (23). This study differs from earlier work published by this laboratory in that in this evaluation we increased the number of pancreases in each of the experimental groups as well all pancreases were processed using the automated methods of islet isolation developed by Ricordi et al. (8). Recently, there was a study that evaluated donor factors in human islet isolation. Although their sample size was small (only 11 pancreases with >9 hours of cold storage), Zeng et al. found a correlation between the duration of cold storage and the post-purification islet recovery (24). As well, the purity of the preparations was significantly reduced as compared to pancreases with <8 hours of cold storage before islet isolation (24).

Analysis of the worldwide experience in clinical islet transplantation by the Islet Transplantation Registry has recently shown that no patient transplanted with isolated islets from pancreases with >8 hours of cold storage has achieved normalized blood glucose levels and insulin independence post-transplant (25).

The ultimate assessment of procurement and storage of the pancreas, is the ability to successfully isolate large numbers of viable and functional islets. Factors that may suggest a suboptimal pancreas procurement include edema of the pancreas, tears in the capsule, or lacerations of the pancreatic parenchyma.

This retrospective study demonstrates that there was no difference in post-digestion recovery, post-purification yield, and functional viability from pancreases procured before or following *in situ* vascular flushing and confirms a randomized trial recently published (10). When examining the post-purification yields it was noted

that with any storage period there was a significant decline in post-purification recovery of islets when compared to control non-stored pancreases. There was a second significant decrease in islet recovery with pancreases stored for more than 16 hours before islet isolation.

The *in vitro* function of the isolated islets showed a cumulative decrease in viability assay results with any increase in duration of cold storage as compared to local control pancreases. This finding was demonstrated both in the actual perifusion curves from islets isolated and in the calculated SI.

Examining both the ability to recover over 100,000 IE and the subsequent function in perifusion of the isolated islets, there was a trend to an increased number of isolations considered to be failures as the duration of cold storage increases. The differences reached statistical significance when the duration of cold storage was > 16 hours of cold storage before islet isolation.

Future initiatives to improve the ability to isolate islets with prolonged cold storage include the development of optimal pancreas cold storage solutions and the establishment of new techniques. Examples of new areas of research with regards to cold storage or preservation before islet isolation include evaluating the actual temperature of organ preservation (27) and developing storage solutions, specific for the pancreas, capable of storage for 24 hours without affecting the Beta cell yield (28). The ex vivo chasing or flushing of the cold storage solution before islet isolation was recently examined. The results demonstrated that organs not chased with Collins-BSA-benzamidine solution resulted in a 50% reduction in Beta cell yield with an increased proportion of damaged cells (29).

CONCLUSION

In consideration of the decreased number of islets isolated from the pancreas, the decline of islet viability with increased duration of cold storage, and the decreased rate of overall success in islet isolations, we have selected 16 hours as the upper limit for cold storage of human pancreases that have been *in situ* flushed with UW solution. We conclude that with the current methods available to recover and store cadaveric donor pancreases and the methods currently used to isolate human islets, it is not an efficient use of resources to attempt to isolate islets from pancreases that have been subjected to cold storage of > 16 hours.

Table III-1:

Islet recovery (> 100,000 IE of > 50% purity) and *in vitro* viability (glucose perifusion, SI >2) from cadaveric donor pancreases loaded with collagenase solution after primary pancreatectomy or pancreases flushed and stored in UW solution before islet isolation.

Group	n	Failed Isolations (n)	Recovery of Purified Islets (%)	Percent Viability	Recovery of Purified Viable Islets (%)
Local Loaded	33	4	88	94	83
Local UW Flush	23	4	83	88	73
UW 3-8 hours	28	4	86	85	73
UW 8-16 hours	49	13	73	73	53
UW >16 hours	13	8	38	25	10

Figure III-1:

Pre-purification islet yields from human cadaveric pancreases as grouped: local pancreases loaded with collagenase solution (Local Load); local pancreases *in situ* flushed with UW solution (Local UW flush); and, distantly procured pancreases subdivided by the hours of cold storage prior to islet isolation (3-8, 8-16, and >16 hours). Islet yield represents mean islet yield (islet equivalents per gram of processed pancreas ±SEM). (* $p<0.05$ vs. Local UW group, # $p<0.05$ vs. 8-16 hour group)

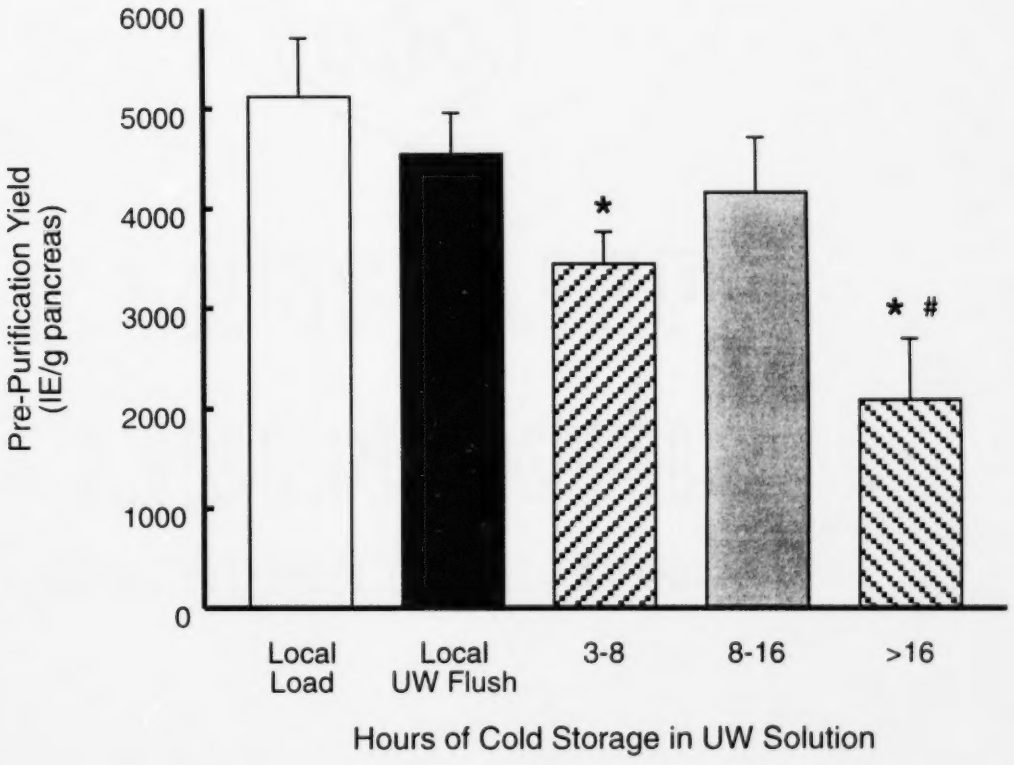


Figure III-2:

Post-purification islet yields from human cadaveric pancreases as grouped: local pancreases loaded with collagenase solution (Local Load); local pancreases *in situ* flushed with UW solution (Local UW flush); and, distantly procured pancreases subdivided by the hours of cold storage prior to islet isolation (3-8, 8-16, and >16 hours). Islet yield represents mean islet yield (islet equivalents per gram of processed pancreas ±SEM). (* $p<0.05$ vs. Local UW group, # $p<0.05$ vs. 8-16 hour group)

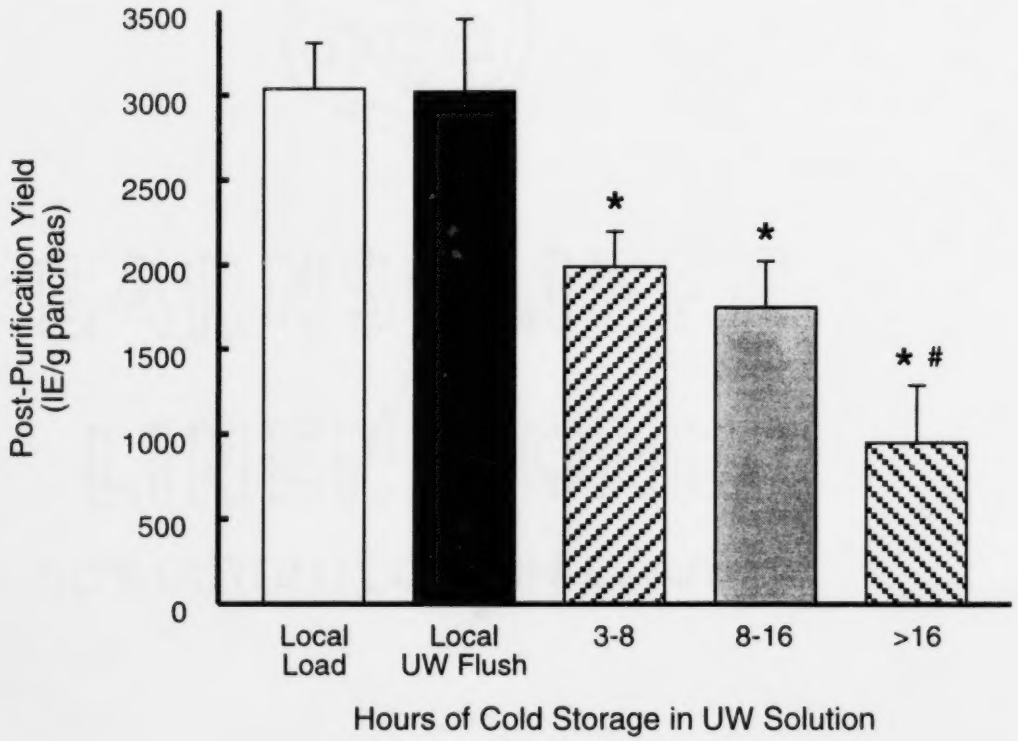


Figure III-3:

Comparison of dynamic *in vitro* insulin secretion from islets isolated from local pancreases flushed with UW solution and processed within 3 hours (Local UW flush) (closed triangle), 3-8 hours (shaded triangle), 8-16 hours (shaded square), >16 hours (open diamond) of cold storage prior to islet isolation as compared to islets isolated from local pancreases removed prior to UW flushing and loaded with collagenase solution (Local Load) (open circle).

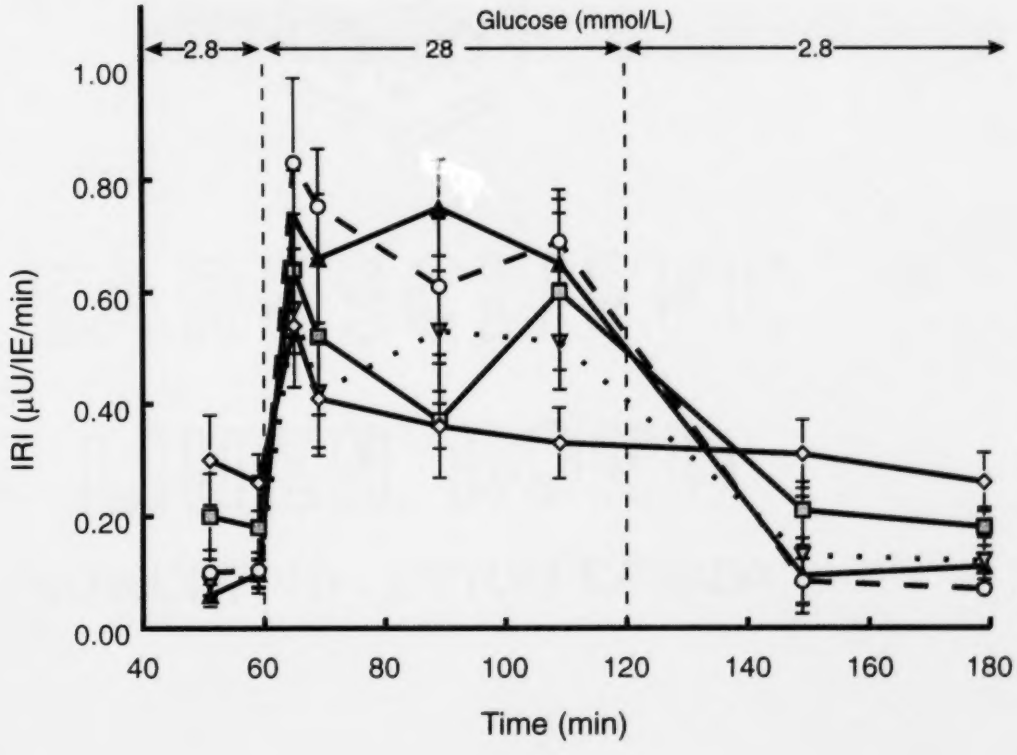
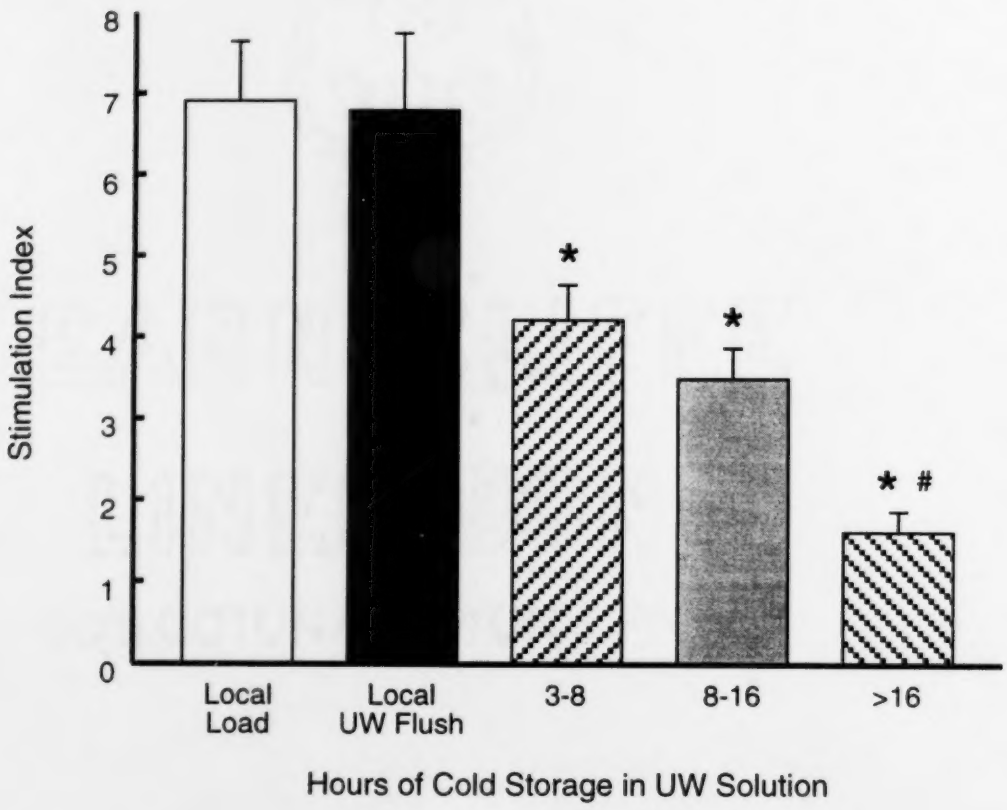


Figure III-4:

Comparison of calculated stimulation indexes from human cadaveric pancreases as grouped: local pancreases loaded with collagenase solution (Local Load); local pancreases *in situ* flushed with UW solution (Local UW flush); and, distantly procured pancreases subdivided by the hours of cold storage prior to islet isolation (3-8, 8-16, and >16 hours).
(* $p<0.05$ vs. Local UW group, # $p<0.05$ vs. 8-16 hour group)



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IV

CADAVER PANCREAS RECOVERY TECHNIQUE: IMPACT ON ISLET RECOVERY AND *IN VITRO* FUNCTION[1]

INTRODUCTION

The procurement of the pancreas is critical to the subsequent ability to isolate and purify large numbers of viable islets of Langerhans (1). Edema of the pancreas or degranulation of the exocrine tissue as a result of a suboptimal procurement of the organ can prevent the successful separation of the islets during the purification phase of islet isolation. Published reports of human pancreas procurement have been largely technical in nature, describing methods for the combined procurement of the pancreas and the liver (2-7). Procurement of the human pancreas for islet isolation has not been specifically addressed.

Rodent studies have suggested that *in situ* vascular flush of the pancreas may result in a decrease in subsequent islet recovery (8, 9). Ohzato et al. showed that *in situ* flushing with Hanks' balanced salt solution reduced the recovery of islets as compared with non-flushed pancreases (8). Our own studies have shown that rodent pancreas procurement incorporating an *in situ* flush can adversely impact the ability to isolate islets depending on the solution infused (9). Ricordi was unable to recover islets from pig pancreases that had been *in situ* perfused with Eurocollins solution (10).

[1] A version of this chapter has been published. Kneteman NM, Lakey JRT, Kizilisik TA, Ao Z, Warnock GL, Rajotte RV. 1994. Transplantation. 58: 1114.

Such published information as well as our own experience with canine pancreas recovery methods (unpublished) led us to utilize initial pancreatectomy before *in situ* flush for our first one hundred local cadaver pancreas procurement procedures (11, 12). This method however, does not easily allow recovery of the entire pancreas, and delays the recovery of other organs. In the present study we compared pancreas recovery by subtotal pancreatectomy before *in situ* vascular perfusion with total pancreatectomy following *in situ* flush in our standard multi-organ procurement technique. We sought to determine whether *in situ* vascular flush with the University of Wisconsin organ preservation solution before pancreatectomy had any adverse impact on ultimate islet recovery or *in vitro* function after collagenase digestion and Ficoll purification with or without subsequent cryopreservation.

MATERIALS AND METHODS

Nineteen consecutive adult cadaveric multi-organ donors were alternately allocated into the two experimental groups. In the primary pancreatectomy group, an incision was made from the suprasternal notch to the symphysis pubis, the sternum split, and an oversized Balfour retractor placed in the abdomen. After thorough laparotomy, the lesser sac was opened by wide division of the gastrocolic omentum. All short gastric vessels were ligated and divided and the spleen and pancreatic tail were mobilized from the retroperitoneum. The splenocolic ligament was ligated and divided. The inferior mesenteric and superior mesenteric veins were identified. The neck of the pancreas was mobilized from the splenoportal venous

confluence. The splenic vein and splenic artery were mobilized and surrounded by 0-silk ligatures. A surgical stapler was fired across the pancreatic neck immediately adjacent to the pancreatoduodenal artery and the pancreas divided with a scalpel. The splenic artery and vein were sequentially ligated and divided, the pancreas removed from the operative field, and the spleen dissected from the pancreas. The pancreatectomy portion of the multiple organ procurement required approximately 30 to 60 minutes.

The *in situ* perfusion group utilized the same standard incision. Following laparotomy, pre-dissection of the hepatic vasculature, and mobilization of the bowels off the retroperitoneum, 30,000 units of heparin were administered intravenously and the aorta cannulated. No portal cannulation nor inferior vena cava cannulation was performed, nor any pre-dissection of pancreas or kidneys. Simultaneous with cardiectomy and/or lung excision an infusion of 3-4 litre of chilled University of Wisconsin (UW) organ preservation solution (Dupont, Wilmington, DE) was carried out via the aortic cannula. The lesser sac was filled with saline slush. The mesenteric vessels were left open and perfused, and secondarily flushed the portal system. The pancreas thus avoids excessive perfusion pressure or restriction of venous outflow. Excessive volume perfusion of the pancreas was avoided by ligating the splenic artery after 1 litre of UW had run through the aortic cannula. The liver and kidneys were excised. The pancreas was then excised from the abdomen with careful dissection from the duodenum to avoid inadvertent entry. The spleen was excised ex vivo. The terminal pancreatectomy required 5 to 10 minutes of careful dissection.

Upon removal by either method, the pancreas was placed in a kidney basin and surrounded by cold (0°C) saline slush. The main pancreatic duct was identified and cannulated with a 14 gauge intravenous cannula (Quik-cath, Baxter, Deerfield, IL) then the pancreas was weighed. One hundred and fifty milliliters of cold (4°C) collagenase solution (type XI, 2.0 mg/mL and type V, 0.5 mg/mL; Sigma Chemical Co., St. Louis, MO) prepared in HBSS (Gibco, Burlington, ON) and sterile filtered using a disposable membrane filtration unit (0.22 μm nylon filter; Corning Inc., Corning, NY). The collagenase solution was then slowly injected into the pancreas using a 60 mL disposable syringe at the back table in the operating room. The same lots of collagenase types XI and V were utilized in all pancreases in this study. The pancreas was then immersed in approximately 100 mL of cold flush solution and packaged in wet ice (4°C) for immediate transport to the islet isolation laboratory for processing within 2 hours.

Upon arrival at the islet isolation lab a 5 mL sample of the transport media was removed for microbiological analysis. The pancreas was placed in a cool cannulation tray where extraneous fat and tissue was removed and the main pancreatic duct was dissected approximately midway towards the tail of the gland. Two 16 gauge intravenous catheters were inserted toward the neck and tail of the pancreas and secured with 3-0 silk ties. The pancreas was then connected to a customized perfusion apparatus (Dr. R.V. Rajotte) and perfused with chilled collagenase solution (2.0 mg/mL Type XI and 0.5 mg/mL Type V) at a pressure of 80 mmHg. Collagenase concentration was arbitrarily increased to 2.2 mg/mL Type XI for the UW perfused pancreases to adjust for possible adverse impact of unknown

components of UW on collagenase activity as has previously been reported (13). After 5 minutes the perfusion pressures were increased to 160-180 mm Hg. At 10 minutes, the collagenase solution was slowly warmed to 35°C. The distended pancreas was cleaned of the outer capsule. The pancreas was then placed in a continuous digestion device (CDD) modified from Ricordi (14). Recirculating collagenase solution was held at 37±0.5°C throughout the dissociation of the pancreas. The endpoint of the isolation was determined by evaluating samples of tissue during the digestion. When the majority of the islets were free from exocrine contaminants the digestion was ceased and warm (37°C) HBSS supplemented with 10% newborn calf serum (Hyclone, Logan, UT) and penicillin/streptomycin (P/S) was flushed through the CDD chamber. The digest was cooled, washed and recombined. An aliquot of the recombined digest was removed and stained with dithizone (Sigma Chemical Co., St. Louis, MO) for pre-purification assessment.

Recombined tissue digest was then purified using discontinuous Ficoll (400-DL, Sigma) gradients which were centrifuged at 550 g for 15 minutes at 22°C. Tissue was removed from the first and second layer interfaces, combined and washed in Medium 199 solution which had been supplemented with 10% fetal calf serum (Gibco, Burlington, ON), 25 mmol HEPES (Gibco) and P/S. The final preparation was brought up to a known volume in CMRL tissue culture media which had been supplemented with 10% fetal calf serum, 25 mmol HEPES, and P/S. The islet mass was quantified and the purity was assessed by two independent observers in accordance to the criteria established at the 1989 International Workshop on Islet Assessment (15). Aliquots of tissue from the final suspension were removed,

incubated with dithizone, and examined microscopically with the aid of an optical graticule. The crude number of islets in each diameter class were counted and then converted into the standard number of islet equivalents (IE, number of islets of 150 μm in diameter equal in volume to sample). Purity was assessed subjectively by comparing the relative quantity of dithizone stained to unstained tissue.

Islets were then cryopreserved using previously reported methods (16). Briefly, aliquots of islets were placed in siliconized Kimex glass tubes and equilibrated with dimethyl sulfoxide (DMSO; Fisher, Ottawa, ON) added stepwise to a final concentration of 2 molar. Islets were supercooled to -7.4°C, nucleated and held for 10 minutes to allow release of the latent heat of fusion, then cooled at 0.25°C/minute to -40°C. Tubes were then plunged into liquid nitrogen (-196°C) for low temperature storage. Tubes containing aliquots of the islet preparation were rapidly thawed (200°C/minute) to 0°C in a 37°C water bath. The intracellular cryoprotectant agent was removed by using a serial sucrose (0.75 M) dilution. Frozen-thawed islets were then washed and cultured in supplemented CMRL solution (Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C before viability assessment.

In vitro islet function of the freshly isolated islets was assessed using a glucose stimulated perifusion system after a minimum of 24 hours in tissue culture (37°C) (17, 18). Islets that were cryopreserved were cultured for 48 hours at 37°C before viability assessment. Aliquots of islets were perifused in duplicate and the insulin content from the perifusion samples was determined using insulin double antibody radioimmunoassay kits (Pharmacia Diagnostica, Uppsala, Sweden). The stimulation

index is a functional index of the insulin secretion by the islets during the high glucose (500 mg/dL) stimulation period over the insulin secreted during both basal periods of low glucose (50 mg/dL) infusion: total of four stimulated insulin secretion rates divided by the total of four (two pre-stimulation and two post-stimulation) basal secretion rates. In addition the total stimulated insulin release following *in vitro* hyperglycemic stimulus was calculated by subtracting the mean basal insulin release from the stimulated release values over the 60 minutes of stimulation. This calculation is graphically illustrated in Figure IV-1.

Statistical Analysis. Results are expressed as mean ± SEM. The data was analyzed by the Student's t-test using the SPSS statistical software package. Significant differences between the experimental groups were assumed if the probability was less than 0.05.

RESULTS

Pancreas Recovery. Donor variables including age, cause of death, body weight, days in ICU, serum amylase, peak and mean blood glucose, and mean dopamine dose administered were not significantly different from each other (Table IV-1). Pancreases procured before *in situ* flush had a mean warm ischemia of approximately 2 minutes (1.8±0.5 minutes) due to the time required to ligate the vessels and remove the pancreas from the donor. The processed pancreas weight before initiating the isolation procedure was significantly lower in the group where the pancreas was procured before the *in situ* flush (66.7±4.0 g vs. 90.6±6.9 g, $p<0.05$). This was expected since a portion of the head of the pancreas was not

removed during the primary pancreatectomy. Following pancreas procurement the isolation of the islets followed the same protocol.

Digestion and Islet Recovery. Following pancreas procurement the isolation of the islets followed the same protocol. The endpoint of digestion as estimated by appearance of the majority of islets well cleared from exocrine tissue occurred at 27.6 ± 1.4 minutes in the nonflushed pancreases and at 40.0 ± 2.0 minutes in the pancreases that had been flushed with UW solution ($p < 0.05$). When the recovery of islets after the digestion phase of the isolation was calculated there was no statistically significant difference between the two experimental groups (Table IV-2). The recovery of islets following Ficoll purification did not demonstrate a significant variation between groups ($70.7 \pm 16.0\%$ in UW flush group vs. $63.8 \pm 8.9\%$ recovery in primary pancreatectomy, p = NS), although a trend to increased total islet recovery in the UW flush group was apparent ($217 \pm 41 \times 10^3$ IE vs. $181 \pm 25 \times 10^3$, p = NS).

In vitro Viability Assessment. Islets isolated from both the *in situ* vascular flushed group and islets isolated from pancreases procured before *in situ* vascular perfusion released insulin appropriately in response to a hyperglycemic stimulus (Figure IV-2). The stimulation index values from islets recovered by both techniques and studied before or after cryopreservation are given in Table IV-3. No significant differences were noted. Similarly no statistical variation was observed in the total stimulated insulin release (stimulated area under the curve of insulin release, Table IV-3).

Sterility. One pancreas in each group was proven to be contaminated after recovery. Cultures grew coagulase(-) staphylococci from the transport media

surrounding the pancreas removed by primary pancreatectomy and Gram(-) bacilli from the *in situ* flush pancreas transport media. Samples taken at the end of the islet isolation procedure were negative in all cases.

DISCUSSION

During previous rodent studies in our laboratory which were designed to evaluate the effectiveness of various solutions in pancreas preservation before islet isolation, an apparent adverse impact of *in situ* vascular flush on subsequent recovery and function of islets was noted (9). Similarly, Ohzato has published data in a rat model demonstrating decreased islet recovery following *in situ* flush (8). Ohzato noted a dramatic drop when HBSS was utilized for the aortic flush and a less obvious drop when modified Sacks solution was utilized (8). Our own studies demonstrated a substantial drop in islet recovery with either Euro-Collins or UW solution flush with no significant alteration when Ringer's lactate was utilized for the initial flush (9). These variations with differing flush solutions are important since pancreas recovery for islet isolation must in most cases allow for a satisfactory period of cold storage before pancreas processing. Ricordi studied pancreas recovery in a pig model and demonstrated a dramatic fall in islet recovery following *in situ* flush with Eurocollins solution (10).

The UW solution has rapidly become a near universal solution for *in situ* flush for multi-organ harvesting. Concerns have been raised however about potential impact of the UW solution on collagenase activity and a subsequent ability to isolate islets (19). Casanova et al. have also recently published data suggesting an adverse

impact of UW solution on subsequent islet isolation when it was introduced into the pancreatic duct after pancreas procurement (20). The above controversies in regards to technique of pancreas procurement and impact of UW solution on subsequent islet isolation led us to examine two clinically relevant models of pancreas procurement for islet isolation.

No significant differences in relevant donor variables were noted between the two sequential randomized groups of patients subjected to primary pancreatectomy or *in situ* flush with UW solution before islet isolation and purification. As expected an increased weight of pancreas was processed following *in situ* flush and total pancreatectomy in comparison to the subtotal pancreatectomy carried out before *in situ* flush. This is an important limitation of the non-flushed isolation procedure. An additional consideration is that of safety - primary pancreatectomy is associated with increased potential for surgical mishap during the pancreatectomy at which time loss of other organs could occur. This risk would be increased if total pancreatectomy were carried out before *in situ* flush in an attempt to recover the pancreatic head.

We noted that digestion required a significantly longer period of exposure to collagenase when pancreases had been flushed with UW, despite a 10% increase in concentration of Type XI collagenase in this group. These findings support an adverse impact of UW solution on collagenase activity as has been reported previously by ourselves (12) and others (13). Our own rodent studies suggested that allopurinol may be the component responsible (12).

Nevertheless, our study results demonstrate no difference in the number of islets recovered per gram of pancreas either after isolation or subsequent purification.

With the increased weight of pancreas processed in the *in situ* flush group, there was a trend to increased total islet recovery post-purification; the difference did not achieve significance. *In vitro* evaluation of islet function appeared equivalent after either *in situ* flush or primary pancreatectomy. Islets from all groups when stimulated with a high glucose solution *in vitro* demonstrated the typical biphasic pattern of insulin release with return to basal insulin secretion levels upon completion of the high glucose stimulatory period. When evaluated either by stimulation index of peak to basal insulin release or by total stimulated insulin release as expressed by the incremental area under the curve of insulin release, no significant differences were seen in insulin release *in vitro*.

Our own clinical trials of pancreatic islet transplantation have utilized banked cryopreserved islets to serve as a supplement to freshly-isolated islets and thus achieve a critical mass of islets for transplantation. This approach has achieved prolonged insulin independence after clinical islet transplant (21). We therefore evaluated islets obtained by both procurement techniques again after subjecting them to a freeze-thaw cycle as is inherent in our clinical transplant protocol. Again as demonstrated in Figure IV-2 and Table IV-3 there was no significant difference in *in vitro* islet function.

Delaying pancreatectomy to the end of the organ recovery after opening of the gallbladder, and division of the bile duct and ureter, could increase the risk of pancreatic contamination. We saw no difference in the incidence of bacterial contamination during the organ recovery procedure. The 10% incidence of

contamination in this study is in keeping with previous experience with cadaveric organs (22, 23).

CONCLUSION

This study demonstrates that significantly less pancreas is recovered for islet isolation by primary pancreatectomy in comparison to total pancreatectomy following *in situ* flush. *In situ* flush with UW solution led to a need for extended digestion time during the subsequent islet isolation. The overall recovery of purified islets was not significantly different between the two experimental groups. There were no significant differences in insulin release in response to high glucose stimulation *in vitro*. These results conflict with published results in both small and large animal models and indicate that large numbers of viable islets can be isolated from cadaver human pancreas utilizing either procurement technique. The advantages of decreased operative time, increased safety, and technical ease of procurement favor the *in situ* flush technique. We confirmed an adverse impact of UW solution introduced as *in situ* vascular flush on subsequent isolation of islets from human pancreas, but demonstrated that adjustment of the digestion period (as guided by microscopy of the digestion process) yielded equivalent islet numbers.

TABLE IV-1:

Donor characteristics from pancreases procured following primary pancreatectomy or *in situ* vascular flushing with UW solution.

A.

Group[a]	Age (years)	Body weight (kg)	Serum amylase (IU/L)	Mean blood glucose (mmol/L)
UW Flush	40±4.9	69.5±3.6	76.8±27.7	9.6±1.7
No Flush	41±4.7	68.7±2.3	59.6±12.5	7.8±1.5

B.

Group[a]	Peak glucose (mmol/L)	Mean dopamine (μg/kg/min)	Warm ischemia (minutes)	ICU days	Intracranial hemorrhage
UW Flush	11.4±2.3	9.3±1.5	0	1.8±0.6	7 of 10
No Flush	11.5±1.8	9.7±1.9	1.8±0.5	1.7±0.6	6 of 9

[a] mean±SEM

TABLE IV-2:

Human islet recovery after primary pancreatectomy or *in situ* vascular flushing with UW solution.

A.

Group[a]	n	Pancreas Weight (g)	Post-digestion	
			Total IE x 10^3	IE/g x 10^3
UW Flush	10	90.6±6.9[b]	333±31	4.0±0.4
No Flush	9	66.7±4.0	315±49	4.5±0.6

B.

Group[a]	n	Post-purification		
		Total IE x 10^3	IE/g x 10^3	Percent Recovery
UW Flush	10	217±41	2.9±0.8	70.7±16
No Flush	9	181±25	2.7±0.3	63.8±8.9

[a] mean±SEM

[b] $p<0.05$

TABLE IV-3:

***In vitro* viability assessment of purified islets after primary pancreatectomy or *in situ* vascular flushing with UW solution.**

Group	Perifusion stimulation index		Area under curve stimulated insulin release (μU/IE)	
	Fresh	Cryo	Fresh	Cryo
UW Flush	7.1 ± 1.4	4.0 ± 1.0	6.8 ± 1.3	2.8 ± 0.9
No Flush	5.9 ± 1.3	2.8 ± 1.4	5.4 ± 1.2	2.7 ± 1.2

Figure IV-1:

Graphic illustration of stimulated area under the curve of insulin release during *in vitro* perifusion. Pre- and post-stimulation basal insulin release is averaged and subtracted from the stimulated insulin release to yield the incremental insulin release resulting from hyperglucemic stimulus over a 60 minute period.

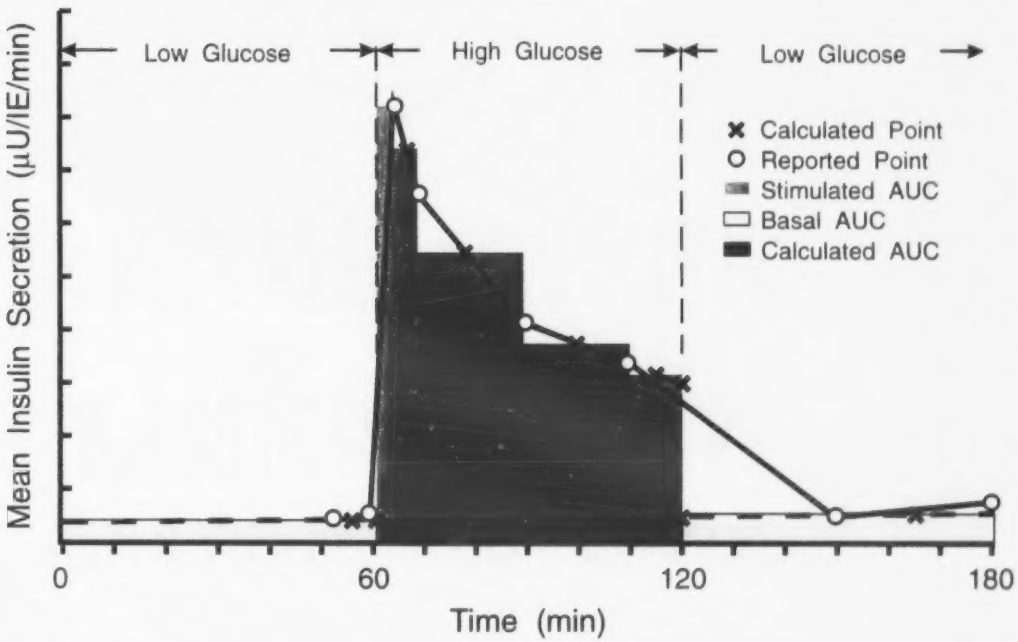
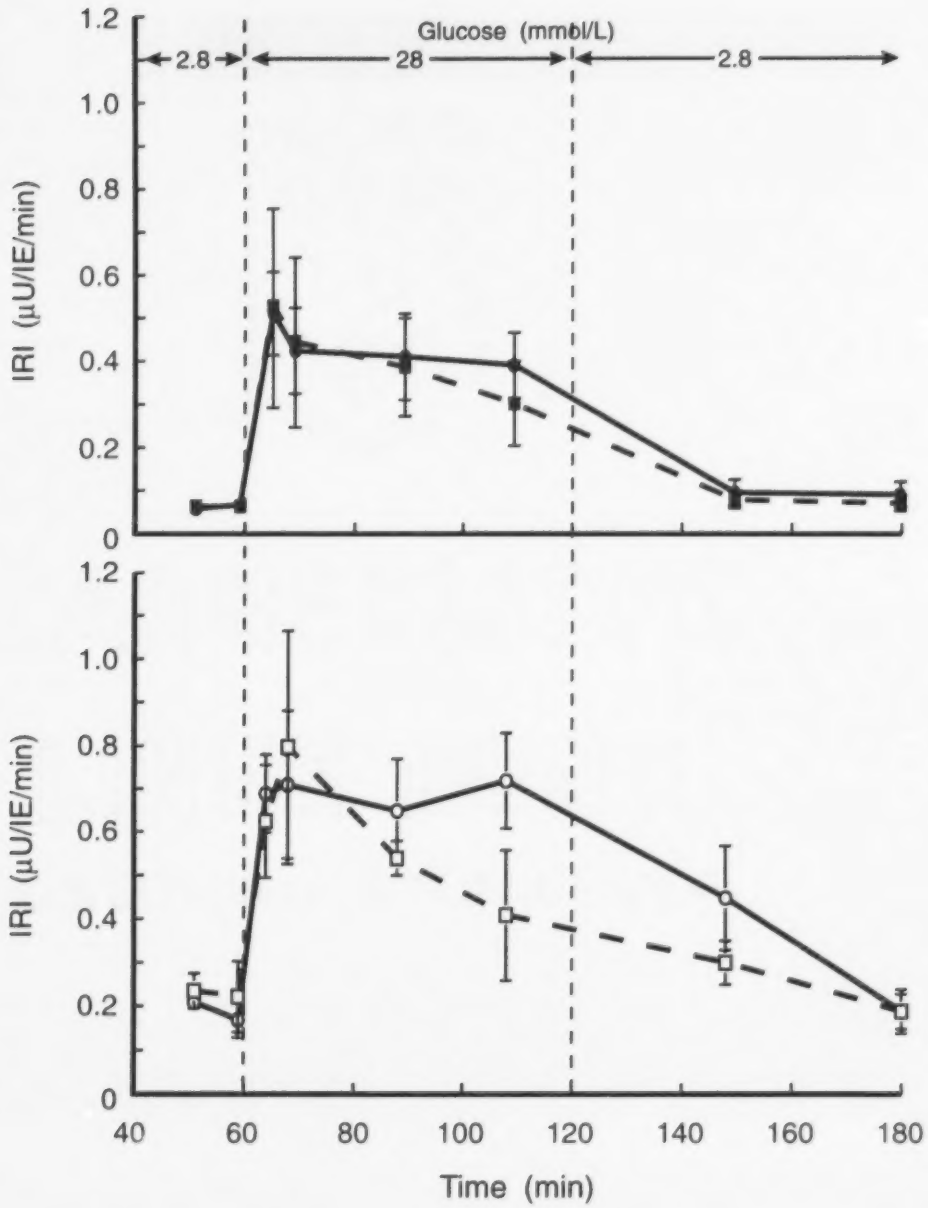


Figure IV-2:

Glucose stimulated perifusion comparing islet function from freshly isolated (upper panel) and cryopreserved islets (lower panel) isolated from pancreases procured before or after *in situ* UW flush. (Nonflushed (■-■, □-□) vs. UW flushed (●-●, ○-○) pancreases)



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V

DEVELOPMENT OF AN AUTOMATED COMPUTER CONTROLLED ISLET ISOLATION SYSTEM[1]

INTRODUCTION

Transplantation of isolated islets of Langerhans into patients with type 1 diabetes has allowed limited numbers of patients to become insulin independent post-transplantation in several centres worldwide (1-5). Recent analysis by the Islet Transplant Registry of 55 pre-transplant C-peptide negative islet allograft recipients between 1990 and 1993 identified several factors that affected the one year islet graft survival (6). Insulin independence was achieved only if greater than 6,000 islet equivalents (IE) per kilogram body weight were transplanted, if islets were isolated from pancreases with a mean preservation time of less than eight hours, islets were transplanted into the liver via the portal vein and if the induction of immunosuppression comprised of T-cell antibodies including antilymphocyte or antithymocyte globulin (6). Current methods used in the separation of human islets incorporate the introduction of collagenase enzyme via the pancreatic duct, followed by enzymatic and mechanical dissociation (7). Digested tissue particles are then separated from the exocrine component using isopycnic density gradient purification (8). For islet transplantation to become a viable and effective therapy, both the quantity and quality of islets being isolated must be improved.

[1] A version of this chapter has been submitted for publication. Lakey JRT, Warnock GL, Brierton M, Ao Z, Hering BJ, London NJK, Ricordi C, Corbin F, Rajotte RV. 1995. Cell Transplantation.

We have developed the Automated Cell Extraction System (ACES) which provides a standard and controlled method for islet isolation (Figure V-1). The ACES system utilizes the concept of the standard continuous digestion device (CDD) originally described by Ricordi et al. where pancreatic tissue is mechanically and enzymatically dissociated in a chamber system (9). This system consists of a lower stainless steel cylindrical chamber containing seven glass marbles (1 cm in diameter) and an upper conical portion (9). A 350 μm stainless steel screen separates these two components and prevents large tissue particles from escaping until they are sufficiently dissociated (9). Using this approach, once sufficient numbers of free islets are identified, the digestion process is ceased and the digested pancreatic tissue containing free islets and the exocrine tissue is collected and recombined using several centrifugation steps (Figure V-2). In the ACES system, we have expanded the automation of the CDD using a pre-programmed personal computer which is able to control and record the steps of the digestion and recombination processes. Once the digestion process is complete, dissociated tissue is passed along a length of flat PVC tubing and allowed to sediment at unit gravity, in a particle collection system which functions as a sluice (Figure V-3). The basic principle of the sluice is that larger tissue particles sediment more quickly than the smaller particles at unit gravity. Sedimented tissue particles are collected in a single intravenous collection bag while the remaining particles and tissue fragments, of approximately <50 μm in diameter, and the bulk of the dilution solution are collected into a waste bag. This new method of tissue recombination eliminates the need for several centrifugation steps and allows recombination of all the tissue in one small collection bag.

It was the aim of this report to describe the initial design and testing of the ACES system and provide experimental data from a series of canine islet isolations where alternate canine pancreases were processed using our standard islet isolation protocol or the ACES system. Both the quantity and the quality of the islets being isolated were assessed to determine the impact of the isolation method on post-isolation survival and function.

MATERIALS AND METHODS

Development of the ACES System. The ACES system was designed as a collaborative project between four research laboratories (Universities of Alberta, Giessen, Leicester and Miami) with manufacturing assistance from the Research and Development Division of COBE-BCT Laboratories (Lakewood, CO). The ACES system consists of a personal computer with an internal driver card, digital and analog boards which control the two dual roller pumps and six tubing divert valves. The computer program monitors the pressure and temperature in the system using an in line pressure transducer (CDX, COBE-CV, Aavada, CO) and four temperature probes (YSI series 400 temperature probes). Accessory boxes (which control the waterbath), solenoids (which control water flow circuit) and the wrist action shaker (Burrell, Pittsburgh, PA) are connected and interfaced to the ACES system. The software program specifically written for the ACES system interfaces the personal computer to the console panel. The software controls the valve position, the tubing pumps, accessory boxes, and provides the user with an extensive history file of each

isolation detailing the exact temperatures, flow rates, and line pressures during each step in the isolation process.

The single use disposable tubing set contains all of the necessary PVC tubing, the in line pressure monitoring device and the in line temperature probes. As well, we have incorporated a disposable heat exchanger into the tubing set. The tubing set also contains all of the required sterile bags for the collagenase and dilution solutions. Having one complete sterile package will reduce the exposure to ambient air, thereby reducing the risk of contamination.

Initial design testing was performed using pancreases from 25 outbred mongrel dogs. All animals were cared for in accordance to the recommendations of the Canadian Council on Animal Care with consultation from a veternarian. Under general anaesthesia the pancreas was mobilized (through an upper midline ventral incision) with all major vascular connections maintained (11). Before excision of the pancreas, cannulas (PE-90) attached to 23 gauge needles were inserted into the right and left branches of the main pancreatic duct. Following excision the pancreas was immediately cooled to 4°C in cold Hanks' balanced salt solution (HBSS; Gibco, Burlington, ON) and weighed. Fifty millilitres of cold HBSS solution containing collagenase (Type V, Sigma; St. Louis, MO) at a dose of 2 mg/mL was injected into both main duct cannulas. A cutdown was performed on the duct and a third cannula was placed approximately 5-8 cm from the distal end of the gland and an additional 50 ml of collagenase solution was injected. The gland was transported to the isolation laboratory where each cannula was connected to a recirculating perfusion device and 4°C collagenase solution was perfused at 300 mmHg (10). After 10

minutes, the temperature was slowly warmed to 37°C over a period of 7-10 minutes. Perfusion was continued until the pancreas appeared soft, then it was transferred to a glass shaker chamber (11, 12) where it was dissociated using established protocols (9, 12). The ACES system has been developed to automatically prime the recirulation circuit with collagenase solution and bring the temperature of the system up to 37°C. A single computer command automatically increased pump speed to 300 mL/minute and increases the shaking velocity 20 seconds before sampling. This control of the sampling sequence allows a more consistent sample of the tissue digest to be examined. Samples of the tissue digest are automatically taken every two minutes throughout the dissociation process. When sufficient numbers of free dithizone-stained islets (13) were identified from these tissue samples, the digestion phase was ceased and the CDD chamber was flushed with 6-8 L of HBSS which had been supplemented with 2% newborn calf serum (NCS). Advancing from the digestion to dilution phase required a single command from the operator which automatically switched the valves and pumps. Additionally, the ACES system utilizes a novel method of tissue recombination. During the dilution phase, as tissue is released from the CDD it passes over the sluice column which allows time for tissue particles to sediment at unit gravity along a flat length of PVC tubing. Sedimented tissue particles are collected from the bottom of the sluice in a single intravenous bag (Baxter, Deerfield, IL) and kept on ice (collection). All remaining liquid and tissue which has not sedimented in the sluice is routed into two 4 L capacity PVC bags (waste). The volume and quantity of tissue in both the collected and waste bags were recorded. For these initial series of experiments the tissue in the waste bag was

drained into 250 mL centrifuge tubes, centrifuged at 450 g for 60 seconds and the tissue recombined to determine the percentage of islet and exocrine tissue losses in the sluice. Tissue digest from both the collection and waste bags was placed in 250 mL flasks for post-digestion evaluation. Duplicate aliquots of tissue were removed from each group, stained with dithizone (13, 14) and the diameter of each islet was sized and categorized using a graticule in the eyepiece of a dissecting microscope. The islet mass was quantified and converted to the number of islet equivalents (IE) in accordance with the criteria established at the 1989 International Workshop on Islet Assessment (15).

Standard Isolation Protocol vs Automated System (ACES). Following the initial design experiments determining the optimal angle and flow rates of the sluice, pancreases from twelve outbred mongrel dogs were alternately assigned to Group 1, our standard digestion system, or to Group 2, the ACES system (Figure V-4). Pancreas procurement, collagenase lot, and loading technique were kept constant for all twelve isolations. Following excision, the pancreas was cooled to 4°C in HBSS and loaded with collagenase via the duct (Type 17449, Serva, Heidelberg, Germany) at a dose of 2 mg/mL as previously described. Following collagenase perfusion the distended pancreas was placed in the glass dissociation chamber containing seven glass marbles and connected to either the standard tubing set or the ACES system (Figure V-2). The standard system consisted of the glass dissociation chamber connected to our customized glass heat exchanger (RV Rajotte custom design) and the tubing pump (Piper pump, Dungey Inc., Agincourt, ON) with 3/8 inch silastic tubing (Cole-Parmer, Niles, IL). The ACES system consisted of an identical glass

dissociation chamber which was connected to our ACES tubing set. The recirculating collagenase temperature in both systems was maintained at 37 ± 0.5°C throughout the digestion phase. The ACES system was automatically primed with collagenase solution. Tissue aliquots (0.5 to 1.0 mL samples) were automatically collected from the sampling port at fixed intervals and examined under a dissecting microscope after staining with dithizone (13). When the majority of the islets were judged to be free from the exocrine tissue, the digestion process was stopped. The ACES system was capable of automatically switching from the digestion phase to the dilution and collection phase with the press of a single computer key (Figure V-2).

Eight litres of warm (37°C) HBSS solution supplemented with 2% NCS and 100 units/mL Penicillin and 100 μg/mL Streptomycin (P/S) was flushed through the digestion chamber. The effluent tissue and dilution solution was cooled by directing the flow through the heat exchanger. Group 1 tissue and solution was collected in multiple 250 mL round flasks (Corning), centrifuged at 1500 rpm (800 g) and recombined. A total of three spins was required to recombine all of the tissue. Group 2 tissue and dilution solution was automatically routed through the particle collection system (Figure V-3). Sedimented tissue was collected in a single intravenous bag on ice at 4°C. All remaining liquid was routed into two large capacity PVC bags (waste bags) as previously described. Duplicate aliquots of tissue were removed from each group, stained with dithizone (13) and the islet mass quantified as previously described (15).

Tissue digest from both groups was purified using discontinuous gradients of Ficoll (400-DL, Sigma) which had been prepared in Medium 199 solution (Gibco)

and centrifuged at 550 g for 25 minutes (16). Tissue was removed from both the 1.045/1.075 and the 1.075/1.085 interfaces, combined and washed in Medium 199 solution supplemented with 10% fetal calf serum (FCS) (Gibco) and P/S. Duplicate samples of the final preparation were removed, stained with dithizone and the number of islets counted and quantified. The purity of the preparations was estimated by comparing the proportion of dithizone stained to unstained tissue. Known quantities of islets were placed in petri dishes containing CMRL 1066 tissue culture media (Gibco) that had been supplemented with 25 mmol HEPES, 10% FCS and P/S and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The percent islet survival was assessed following a 24 h period of tissue culture.

Static Incubation. To assess the *in vitro* functional viability of the canine islets isolated using the two methods, duplicate samples of known numbers of islets were incubated in RPMI solution (Gibco) that had been supplemented with low (2.8 mmol/L), high (20 mmol/L) glucose or high glucose solution supplemented with 50 mmol/L IBMX (Aldrich, Milwaukee, WI) (17, 18). After 2 h incubation at 37°C in a humidified 5% CO_2 incubator, the supernatant was removed and assessed for insulin content using double antibody radioimmunoassay and human insulin standards (19). The insulin secretory capabilities of the islets were expressed per islet equivalent and the stationary stimulation index was calculated by dividing the insulin output from the high glucose plus IBMX group by the insulin value collected during low glucose incubation.

Transplantation. *In vivo* evaluation of the islets isolated using the ACES system and the standard method was assessed by transplanting an aliquot of 2000 IE under the kidney capsule of 5-7 week old Balb/C nude mice (Banting Laboratories, Seattle, WA) (20, 21). Mice were housed under VAF conditions and were rendered diabetic by a single intravenous injection of alloxan (90 mg/kg, ICN Biomedical, Costa Mesa, CA) (20). Only mice with nonfasting blood glucose values >20 mmol/L were used as transplant recipients.

In vivo Assessment. Following islet transplantation blood glucose was assessed twice a week on tail vein blood obtained by tail cuts using a Companion 2 blood glucose meter (Medisense, Waltham, MA). Grafts were considered successful if the plasma glucose returned to below 8.4 mmol/L.

At 50 days post-transplant, all euglycemic mice were subjected to an oral glucose tolerance test. Three grams glucose/kg body weight (50% solution, Abbott Laboratories, Montreal, PQ) was given via oral gavage into unanesthetized mice which were fasted for 2 hours. Blood samples were taken from the tail vein at 0, 15, 30, 60 and 120 minutes (22). Non-transplanted age-matched normoglycemic nude mice were used as controls for comparative purposes.

Statistical Analysis. Results are expressed as mean±SEM. Differences between the experimental groups were analyzed using unpaired student's t-tests, analysis of variance (ANOVA) using SPSS statistical software (Chicago, IL). Differences were considered significant when $p<0.05$.

RESULTS

Development of the ACES System. The initial design and configuration of the ACES system was evaluated on the system's ability to recover isolated canine islets and prevent excessive tissue loss during recombination using the particle collection system. A single use disposable tubing set was developed to incorporate all steps in the dissociation and recombination of the isolation process. Lengths of PVC tubing were connected to produce a flow pattern which provided maximal efficiency and minimal duplication of tubing.

Table V-1A describes the results of the initial series of experiments where digested canine pancreatic tissue was passed over the sluice at different angles and the loss of tissue into the waste port was determined. In experiments where the angle of the sluice was 10° or 25° the loss of islet tissue into the waste port was 3.8±1% (mean±SEM) and 5.8±1% respectively (p=ns). However, at a 10° angle there was considerable accumulation of tissue digest in the length of the sluice. When the angle of the sluice was increased to 45°, the loss of islet tissue increased to 16.7±3.6% (Table V-1A). This was attributed to the spilling of sedimented tissue into the waste because the position of the waste port was near the bottom of the column. Therefore the angle of the sluice which allowed effective sedimentation of tissue digest without excessive accumulation in the column was 25°.

In a second series of experiments, canine pancreatic tissue digest was passed over the sluice at varying flow rates, at a fixed angle of 25°. At a flow rate of 150 mL/minute minimal losses of tissue occurred (3.5±0.8%) (Table V-1B). However, the duration of time required to pass 8 liters of the dilution solution

through the system made this flow rate impractical. Increasing the flow rate to 225 mL/minute resulted in 5.8 ± 1.1% loss of islet tissue. We observed a significant increase in the loss of islet tissue at a dilution flow rate of 300 mL/minute (19.4 ± 1.8%). Therefore we chose to use a flow rate of 225 mL/minute for all subsequent experiments.

Standard Isolation Protocol vs. Automated System (ACES). Pancreases digested using either the standard system (Group 1) and the ACES system (Group 2), were comparable in donor age, body weight and pancreas weight (Table V-2). The collagenase digestion time was 23.3 ± 1.6 minute for Group 1 vs. 25.2 ± 1.4 minute for glands digested in Group 2 (p = ns, Student's paired t-test).

The recovery of islets following collagenase digestion and Ficoll purification from the two groups is found in Table V-3. Recovery of islets following collagenase digestion and recombination was 90.8 ± 21 $x10^3$ IE for Group 1. In the ACES system digested tissue was passed over the sluice and the islets which were collected in the collection port yielded 99 ± 14 $x10^3$ IE (Table V-3). This was not significantly different from the tissue collected in Group 1. Using the ACES system, we were able to evaluate the loss of tissue into the waste port during tissue recombination. A total of 4.5 ± 0.8 $x10^3$ IE or 4.4 ± 1.4% of the islet recovery was lost to the waste port. Tissue lost during recombination in Group 1 was not evaluated.

Post-purification recovery was not significantly different between the two groups with 56.2 ± 14 $x10^3$ IE recovered from Group 1 and 54.7 ± 11 $x10^3$ from Group 2. Overall islet recovery following purification was equal with a 64 ± 6.1% recovery from Group 1 vs. 55.1 ± 8.1% for Group 2.

To evaluate islet recovery following tissue culture, islets were cultured overnight at 37°C. There was no significant difference in the recovery of islets collected from Group 1 and Group 2 (75.3±6.3% and 86.8±2% respectively). Only 55±6% of the islets collected from the waste port of the sluice were recovered following overnight tissue culture ($p < 0.05$ vs group of islets collected from Group 1).

Table V-4 shows the islet size distribution into standard size categories following collagenase digestion, Ficoll purification and overnight tissue culture. Islets isolated using the ACES system and the standard method had a similar size distribution. The majority of islets collected from both groups were less than 150 μm in diameter. However, there were islets in the larger size categories for both groups. All islets collected from the sluice column were less than 200 μm in diameter with a majority (75.2±4.4%) collected being < 100 μm in diameter (Table V-4). There was no statistically significant difference in the proportion of islets lost following purification and tissue culture between Groups 1 and 2.

Static Incubation. Viability of islets isolated using the ACES system or islets isolated using the standard system is demonstrated in Table V-5. Islets collected from both experimental groups responded to high glucose stimulation (p=ns, n=6 individual experiments run in duplicate). When the islets were exposed to 20 mmol/L glucose solution which had been supplemented with 50 mmol/L IBMX, there was a 8.6-fold increase in insulin output for islets from Group 1 and an 8.8-fold increase for Group 2 (p=ns; Table V-5). Islets collected from the waste port of the ACES system had a significantly higher basal insulin output (Table V-5).

Transplantation. Function of transplanted islets from the two experimental groups in alloxan induced diabetic nude mice is shown in Table V-6. Five out of the seven mice receiving 2000 IE isolated using the ACES system returned to and maintained euglycemia beyond 50 days post-transplant compared with five of six from Group 1. Only one of the five diabetic nude mice which received islets from the waste port maintained euglycemia longterm. Mean blood glucose profiles of the islet transplanted mice are presented in Figure V-5. Removal of the kidney bearing the islet graft in mice surviving >50 days post-transplant resulted in prompt hyperglycemia.

In vivo Assessment. Figure V-6 demonstrates the response to a glucose challenge. Functional assessment of the islet grafts at 50 days post-transplant using an oral glucose tolerance test showed that mice transplanted with 2000 canine islets isolated with either the ACES or standard method responded equally to the glucose load. The peak glucose response for Group 1 was 10.7 ± 0.6 mmol/L and for Group 2 was 11.4 ± 0.6 mmol/L vs non-transplanted control mice which had a glucose peak of 11.4 ± 1.7 mmol/L (Figure V-6).

DISCUSSION

Clinical islet transplantation offers much promise in the treatment of insulin dependent diabetic patients. Currently, it is necessary to pool islets from several donors in order to achieve an islet mass which will allow for the removal of exogenous insulin therapy. If islet transplantation is to become an effective and reliable method in the treatment of diabetes, both the quality and quantity of islets

must be improved. We have developed the ACES system, a computer controlled system that standardizes the pancreas dissociation process providing the opportunity to examine factors in the isolation process which may lead to maximizing islet recovery.

Although the protocols for the isolation of rodent islets are well-established and consistent, initial attempts at using these protocols for the isolation of canine and human islets was ineffective. This may have been due to the compactness and dense connective tissue stroma found in the canine and human pancreas (23). Delivery of the collagenase enzyme via the main pancreatic duct was shown to be an effective method for the cleavage of the islets from the exocrine tissue (24, 25). Controlled delivery of the collagenase using a perfusion device was first described by Horaguchi and Merrell (10). This approach was further refined to allow precise control of temperature and perfusion pressure (12, 26).

Following effective delivery of the collagenase to the islet-exocrine interface, various methods of pancreas dissociation have been attempted including tissue macerators (27) and counter-rotational blades (28, 29). However, the shearing forces created using these methods caused excessive islet fragmentation (23, 24). The next advance was to use a more gentle approach for pancreas dissociation which involved shaking and aspirating the partially digested tissue through various sizes of needles until the islets were free from the exocrine tissue allowing increased recovery of islets following purification (23-25).

In 1988 Ricordi described a closed chamber recirculation system for the isolation of human islets (9). This approach had the advantage of minimal trauma

to the islets and allowed for a continuous digestion process with the collection of free islets as they are liberated from the digestion chamber (9). Since the introduction of this method, many laboratories have utilized this technique to isolate canine and human islets. Although this was called an automated system, the procedure still requires a certain level of expertise to handle the several steps in the isolation process. The ACES system expands on Ricordi's method by adding a truly automated computer based system with a new component for tissue recombination. Additionally, all necessary tubing and supplies can be provided in a single use disposable set. The ACES system was initially designed to allow the loading of pancreas with collagenase solution independent of the dissociation, since many centres prefer manually loading the collagenase solution.

Using standard canine islet isolation protocols, following dissociation tissue digest is recombined using several centrifugation steps. The ACES system incorporates a column of flat one inch width of PVC tubing which we have termed sluice, for tissue recombination. Experiments which evaluated the optimal angle and flow rate of tissue digest into the sluice determined that the loss of tissue into the waste port was approximately 5% when the angle was 25° with a flow rate of 225 mL/minute. Increasing the angle or flow rate resulted in excessive tissue losses. Additionally, the tissue loss into the sluice waste port had an elevated basal insulin secretion during static incubation. Only one of five mice transplanted with islet tissue from the waste port maintained euglycemia for >50 days in alloxan-treated nude mice. During standard isolation procedures there may be a similar loss of islets in the supernatant following centrifugation.

A comparison of the results obtained from a consecutive series of canine islet isolations using either the ACES system or the standard system revealed that the quantity and quality of islets isolated was comparable. Islet morphology was equivalent with the proportion of islets in each size category being equivalent with the exception of smaller sized islets from the ACES system which were lost when the tissue digest was passed over the sluice. These islets appeared to have a reduced viability suggesting that they may be somewhat damaged.

A complete isolation package containing all the necessary tubing, heat exchanger and bags adds simplicity to the system and has an additional advantage of being a closed system which should reduce the likelihood of microbiological contamination. Our series of isolations comparing the ACES system to the standard method utilized the same lot and dose of collagenase. The recovery of islets was below our standard recovery using an effective batch of collagenase, however, the results from the paired series of experiments are still valuable when comparing our experimental groups.

The ultimate goal of the ACES system is to develop a consistent and reliable method for the isolation of human islets. This initial series of experiments in the canine model support evaluation and testing of the ACES system for human pancreas dissociation.

The development of the ACES system offers several advantages to the standard isolation process. By having an isolation system that is easy to use and consistent, this technology and method of isolating islets can be utilized by more centres which have limited experience in the isolation of islets. As well, development

of a standard isolation system allows more control over the isolation process and allows for the potential to examine the isolation process indepth to optimize collagenase efficiency, loading techniques and dissociation of islets. A controlled isolation system requires less reliance on operator experience and can be performed with fewer individuals.

CONCLUSION

It was the aim of this project to describe the development of a more automated method of separating and recombining islets from the canine pancreas. The recovery and post-isolation function of islets isolated using the ACES system was equivalent to current methods. This report also describes an effective method of tissue recombination eliminating the need for several centrifugation steps following pancreas dissociation.

Table V-1:

Islet losses from sluice collection system with varied collection angle and flow rate.

A. SLUICE COLLECTION ANGLE

	Sluice collection angle (degrees)		
	10	**25**	**45**
Percent Islet Loss	3.8 ± 1.0[a]	5.8 ± 1.0	16.7 ± 3.6
Number of Experiments	3	5	4

B. FLOW RATE

	Flow rate into sluice (mL/minute)		
	150	**225**	**300**
Percent Islet Loss	3.5 ± 0.8	5.8 ± 1.0	19.4 ± 1.8
Number of Experiments	4	5	4

[a] mean ± SEM

Table V-2:

Donor and isolation variables for studies of standard vs. automated islet isolation protocol.

Group	n	Donor weight (kg)	Pancreas weight (grams)	Digestion time (minutes)
Standard	6	24.9±0.9	44.5±3.0	23.3±1.6
ACES	6	27.1±2.5	41.1±3.3	25.2±1.4

Table V-3:

Yields of canine islets isolated with the standard or the ACES system.

Group	Post-Digestion (x 10^3)		Post-Purification (x $10^{3)}$		Percent Recovery
	Total IE	IE/gm	Total IE	IE/gm	
Standard	90.8±21.0	2.2±0.6	56.2±14.0	1.4±0.4	64±6.1
ACES Collection	99.0±14.0	2.5±0.4	54.7±11.0	1.4±0.3	55.1±8.1
ACES Waste	4.5±0.8	0.1±0.02	2.9±0.4	0.07±0.01	80.7±16.3

Table V-4:

Islet size distribution following collagenase digestion, Ficoll purification and following 24 hour *in vitro* tissue culture.

A. Standard Isolation Method

	Islet size distribution, μm (% of islets)				
	55-99	**100-149**	**150-199**	**200-250**	**>250**
Post-digestion	46.5±3.6	41.8±2.8	8.5±1.4	3.0±0.9	0.2±0.2
Post-purification	39.5±6.0	46.2±4.9	13.0±2.1	2.7±0.6	0.2±0.2
Post 24 hour culture	44.7±6.2	39.2±5.5	11.7±2.6	2.1±0.4	0.1±0.1

B. ACES - Collection

	Islet size distribution, μm (% of islets)				
	55-99	**100-149**	**150-199**	**200-250**	**>250**
Post-digestion	51.3±5.0	34.3±3.7	11.0±1.9	2.7±0.3	0.5±0.3
Post-purification	36.2±4.2	45.7±4.2	13.7±0.7	4.0±0.5	0.8±0.4
Post 24 hour culture	44.2±6.1	39.0±4.7	14.4±2.0	2.6±0.4	0.2±0.2

Table V-4:
Continued

C. ACES - Waste

	Islet size distribution, μm (% of islets)		
	55-99	**100-149**	**150-199**
Post-digestion	75.2±4.4	22.0±5.0	2.7±0.6
Post-purification	65.7±5.3	30.5±6.7	3.7±0.7
Post 24 hour culture	68.3±6.3	26.2±5.1	5.7±1.2

Table V-5:

Insulin response of isolated canine islets during static incubation.

	Insulin secretory activity (μU/IE/h)			
	2.8[a]	**20**	**20 + 50 mmol/L IBMX**	**Mean SI**
Standard method	2.1 ± 0.4	6.4 ± 0.6	18.2 ± 1.0	8.6x[b]
ACES: Collection	2.6 ± 0.6	5.5 ± 0.9	22.8 ± 2.1	8.8x
ACES: Waste port	5.6 ± 0.5	13.9 ± 1.8	23.4 ± 6.0	4.2x

[a] mmol/L glucose

[b] Stimulation Index (20 mmol/L glucose + 50 mmol/L IBMX divided by 2.8 mmol/L glucose)

Table V-6:

***In vivo* function of alloxan-induced diabetic nude mice transplanted with canine islets isolated using either the standard isolation protocol or isolated using the ACES system.**

Group	Days of normoglycemia (median)	Percent graft survival at 50 days
Standard isolation method	0x2, >50x5 (**>50**)	71%
ACES system - Collection	0,10, >50x5 (**>50**)	71%
ACES system - Waste port	0x2,5x2, >50 (**5**)	20%
Non-transplanted diabetc controls	4,8x2,10x2 (**8**)	0%

Figure V-1:

The Automated Cell Extraction System during the recirculation phase of the islet isolation process.

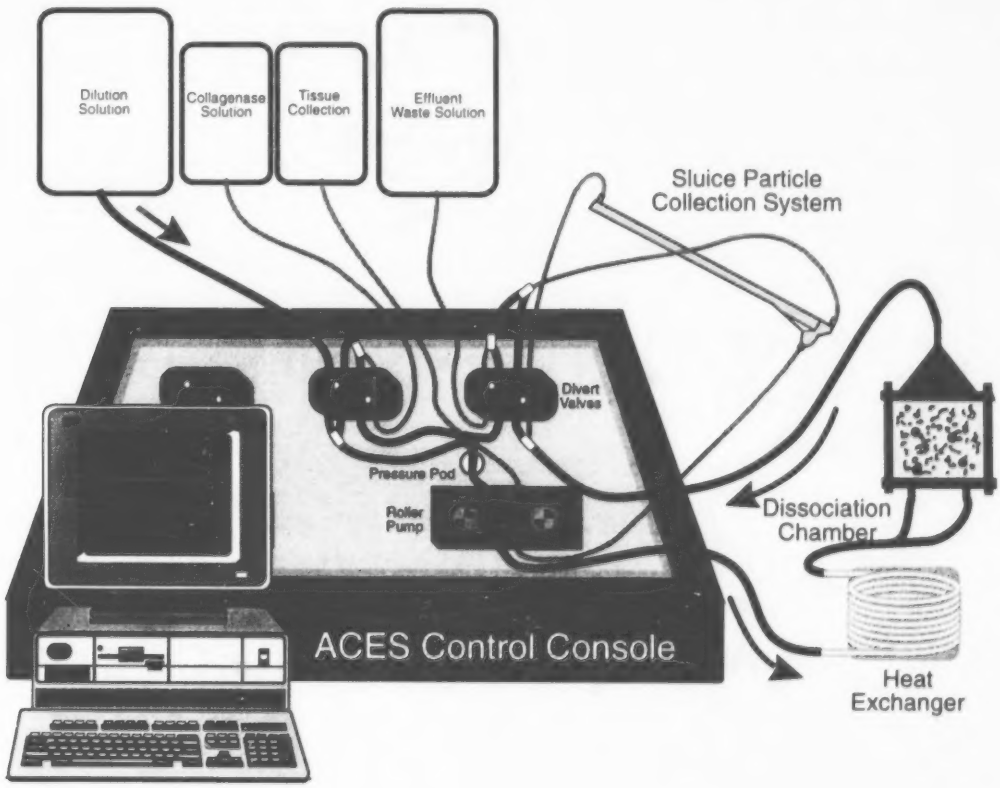


Figure V-2:

The Automated Cell Extraction System during the dilution and collection phase of the islet isolation

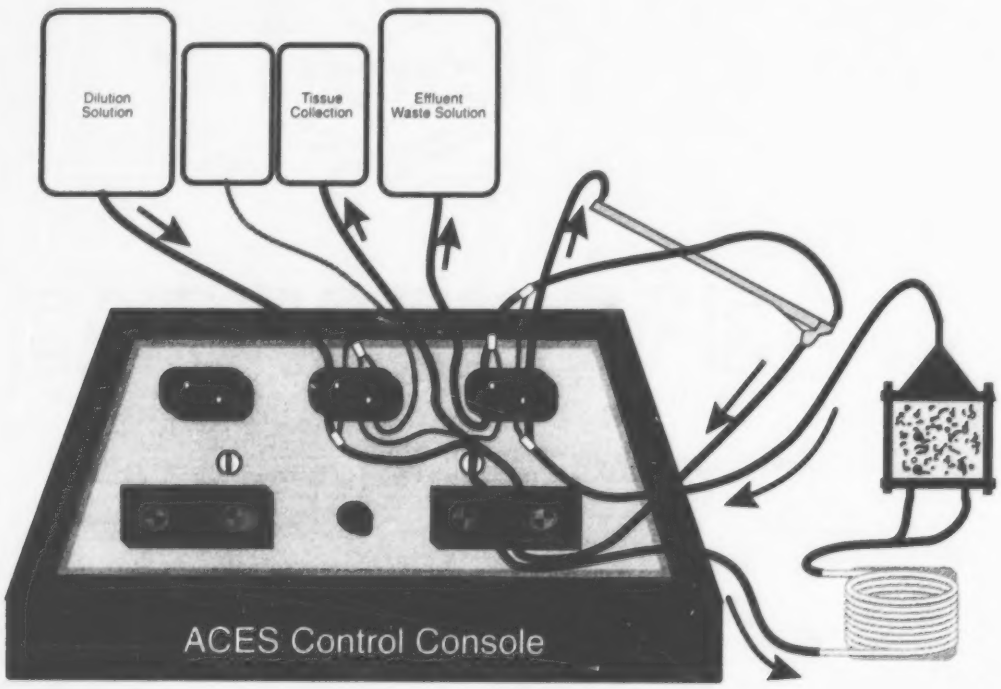


Figure V-3:

Particle collection system used to collect digested pancreatic tissue fragments along a flat length of PVC tubing. Tissue particles are allowed to sediment and are collected into a single intravenous bag on ice. All remaining particles and the majority of the dilution media are vented into the waste port and collected in two large capacity bags.

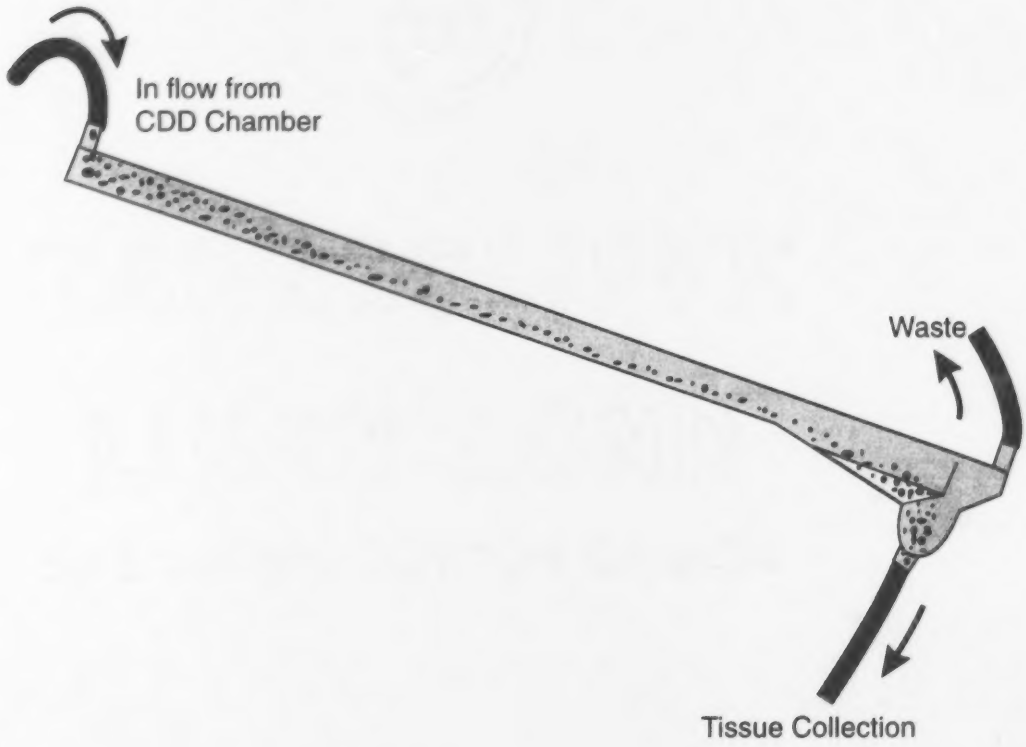


Figure V-4:

Experimental protocol for the isolation of canine islets using either the standard method or using the ACES system.

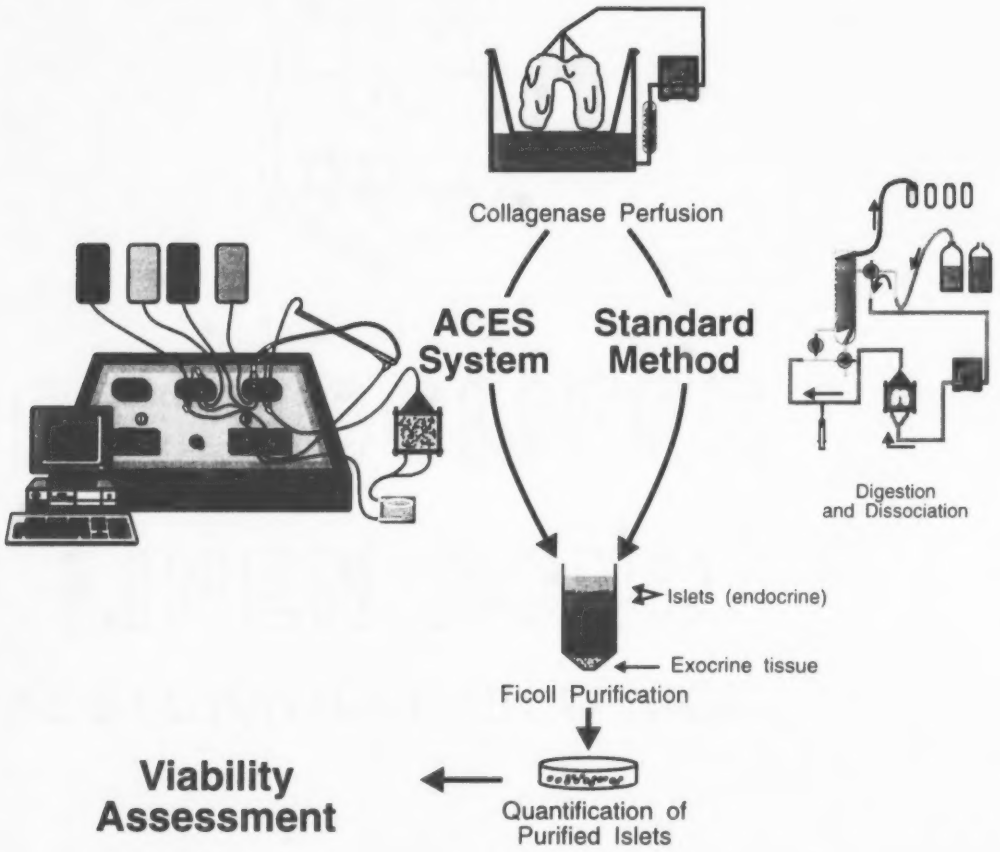


Figure V-5:

Blood glucose profiles of alloxan induced diabetic Balb/c nude mice receiving 2000 isolated canine islets using the standard method (closed box), from the collect port of the ACES system (open circle) or from the waste port of the ACES system (open triangles).

Arrows = failed transplants (blood glucose >15 mmol/L for two consecutive readings).

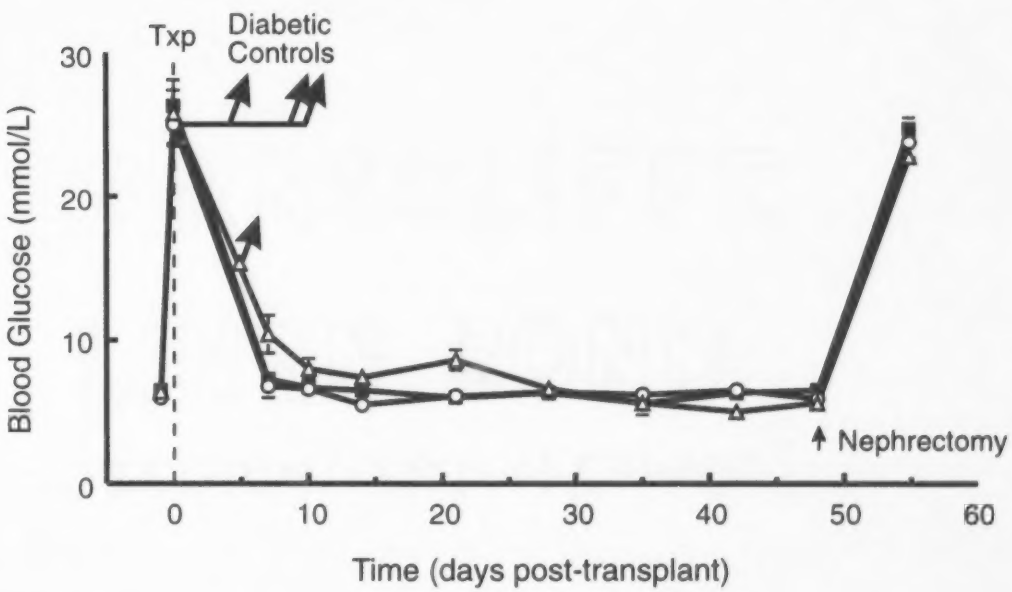
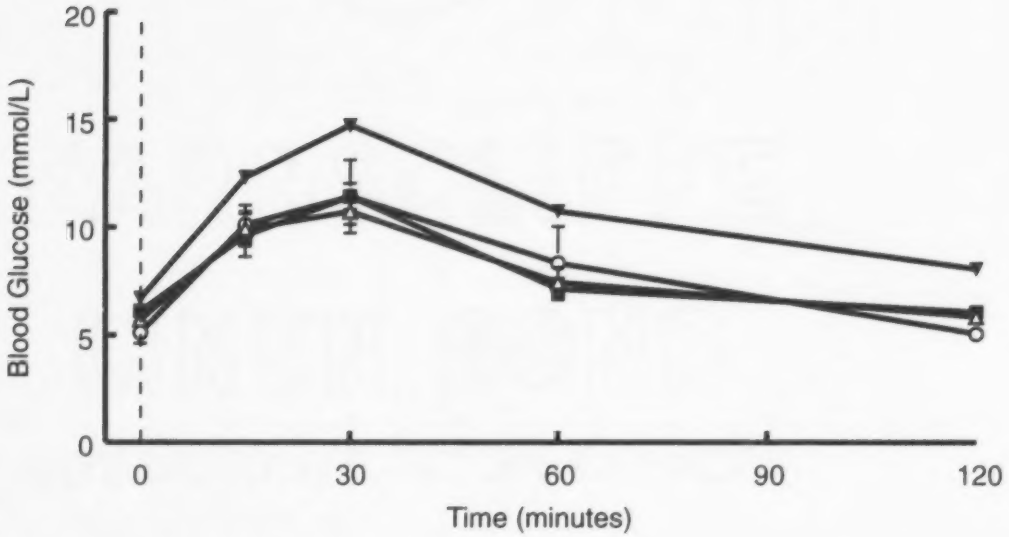


Figure V-6:

Oral glucose tolerance test of alloxan-induced diabetic nude mice transplanted with canine islets isolated using the standard isolation method (closed box), from the collect port of the ACES system (open circles) or from the waste port of the ACES system (closed triangles) as compared to non-transplanted age matched control animals (open triangles).



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VI

MICROBIAL SURVEILLANCE OF HUMAN ISLET ISOLATION, *IN VITRO* CULTURE, AND CRYOPRESERVATION[1]

INTRODUCTION

Transplantation of isolated islets of Langerhans has been shown to induce long-term normoglycemia and insulin independence in type 1 diabetic patients (1-3). Evaluation of recent clinical islet transplants by the International Islet Transplant Registry has identified critical conditions necessary, including transplantation of >6,000 islet equivalents/kg body weight, to increase the potential for insulin independence post-transplant (4). Limitations in the isolation of viable islets from cadaveric donor pancreases make it necessary to collect pools of isolated islets from several donors to achieve this critical islet mass. Tissue banking through short-term tissue culture and long-term banking through cryopreservation are the methods currently used to achieve this. These methods of islet isolation and storage can create the potential risk of microbial contamination and recommendations have been made for surveillance (5-7). Transplantation of microbiologically contaminated islets into immunosuppressed patients has been recently documented (8).

In the current methods of islet isolation and islet culture there are multiple steps where microorganisms could potentially infect the islet preparation. Furthermore, the use of islets from multiple donors can compound the risks of infusion of contaminants into individual recipients. No reports have documented the

[1] A version of this chapter has been published. Lakey JRT, Rajotte RV, Warnock GL. 1995. Clin Invest Med. 18: 168.

mechanism for microbiological contamination nor the frequency of positive microbial cultures of islets maintained in a low temperature tissue bank. The aims of this study were first to retrospectively evaluate the frequency of positive microbial culture of islets isolated and maintained in a low temperature (-196°C) tissue bank, and second to prospectively evaluate the appearance and natural history of microbiological contamination during various steps in the isolation, *in vitro* culture, and cryopreservation of isolated human islets.

MATERIALS AND METHODS

The isolation, *in vitro* tissue culture, and cryopreservation of human islets were performed in a sterile laboratory environment separated from any rodent or large animal experiments (9-11). Microbiological samples were collected aseptically by removing a 2 to 5 mL sample at each specific step. Samples collected during the islet isolation and subsequent cryopreservation were coded for identification and subjected to routine aerobic culture and sensitivity testing by a clinical microbiology laboratory.

One hundred and seventeen pancreases were obtained, with consent, from brain dead cadaver donors as part of multiorgan procurements. Forty-seven pancreases were included from the retrospective study and the remaining seventy comprise the pancreases evaluated in the prospective study. Following procurement, the pancreas was surrounded with cold preservation solution, placed in two sterile plastic bags, and transported to the islet isolation laboratory.

From the seventy pancreases that were included in the prospective study, thirty-two were procured locally and processed within 3 hours of procurement. Nine

of these pancreases were removed before vascular flushing *in situ* with hypothermic preservation solution, and the remaining twenty-three local pancreases were removed as part of a multiorgan procurement after vascular flushing of cold University of Wisconsin (UW) organ preservation solution (12). The remaining thirty-eight pancreases were received from distant collaborative centres and were shipped on ice in UW solution after vascular flushing with UW solution. These pancreases had a period of cold storage ranging from 6 to 20 hours before islet isolation.

For the prospective study, the protocol that was followed for seventy consecutive islet isolations and the groups of samples submitted for microbiological evaluation is detailed in Figure VI-1. Upon arrival at the human islet isolation laboratory, a 5 mL sample of the transport media was removed for microbiological analysis (Step 1). Collagenase solution was injected into the pancreatic duct and perfused for 15 minutes, then a sample of the perfusion solution was collected (Step 2). The pancreas was immersed in a closed chamber, surrounded by collagenase solution, and subjected to further digestion and dissociated by shaking (13). A sample of the tissue digest was removed during dilution phase for microbiological analysis (Step 3). The tissue digest in collagenase solution was washed with Hank's balanced salt solution (HBSS) that had been supplemented with 2% newborn calf serum (Gibco, Burlington, ON) and penicillin (100 U/mL) and streptomycin (100 μg/mL). The dissociated pancreatic tissue was then recombined and the islets were separated from the exocrine tissue by centrifugation in a discontinuous gradient of Ficoll (DL-400, Sigma, St. Louis, MO) (14, 15). After purification, the collected islet tissue was then washed with Medium 199 solution

(Gibco), with 25 mM HEPES (Gibco), supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), and penicillin and streptomycin. After the final wash step, the islets were recombined and aliquots of tissue were removed for counts of islet yield, an aliquot was also removed post-isolation for microbiological assessment (Step 4).

Following islet isolation, all purified islets were placed in sterile tissue culture plates (100x15 mm) (Fisher Scientific, Ottawa, ON) with sterile filtered (0.22 μm) CMRL 1066 tissue culture media (Gibco), supplemented with 10% heat inactivated fetal calf serum (Gibco), 25 mM HEPES and penicillin and streptomycin. Following 6 to 48 hours of tissue culture, *in vitro* islets were recombined. An aliquot of tissue was removed for microbial analysis (Step 5). Duplicate aliquots were also removed for precryopreservation recovery counts.

Islets were cryopreserved using established protocols (16). Briefly, aliquots of islets were placed in siliconized glass Kimex tubes (15 mL) and equilibrated with dimethyl sulfoxide (DMSO) added stepwise to a final concentration of 2 M. Islets were supercooled to -7.4°C, nucleated, and, following the release of latent heat of fusion, cooled at 0.25°C/min to -40°C. Tubes were then plunged into the liquid phase of liquid nitrogen (-196°C) and stored at this temperature while immersed. As part of our routine evaluation of the function and microbiological sterility of our cryopreserved islet preparation, one tube of cryopreserved islets from the preparation was randomly selected from the batch. This tube was rapidly thawed (200°C/min) to 0°C in a 37°C water bath using previously described protocols (16). Immediately after thawing, approximately 2 mL of the supernatant was removed for

microbiological assessment (Step 6). After serial dilution in hypertonic (0.75 M) sucrose solution, a further sample of the frozen-thawed tissue was removed for analysis (Step 7).

Frozen-thawed islets were plated in petri dishes with 10 mL of CMRL media and 10% fetal calf serum and cultured for 48 hours at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide before viability testing using a glucose perifusion system (17, 18). Aliquots of the residual tissue suspension and culture media at the conclusion of the procedure were removed for microbiological analysis (Step 8).

In six cases, two additional tubes containing only the freeze solution were cryopreserved with the islet preparation. The first blank tube was thawed and assessed for microbial contamination when a tube from the islet preparation was thawed for microbial and functional assessment. The second blank tube remained immersed in liquid nitrogen for >3 months, after which it was thawed and subjected to microbiological analysis.

All microbiological samples were collected by aseptic aspiration of 2 to 5 mL of solution in which the tissue was washed or incubated during the above steps. The samples were removed to sterile evacuated flasks and submitted to the Department of Medical Microbiology & Infectious Diseases for routine aerobic culture and sensitivity testing. Samples were coded for identification and then aliquoted into sterile blood agar plates and incubated at 35°C for an initial period of 48 hours. Cultures are quantified as 1+ and 4+ according to streak quadrant of the plates on which they grew. If no growth occurred after a 48 hour culture period, the culture

was reported as negative. If there was growth on the plates, all organisms were identified. In addition to the primary report, an additional report was sent to the Infection Control Department for monitoring of the clinical islet isolation program.

Gram-negative organisms were identified using an automated Vitek system or by conventional biochemical systems if the automated system failed to provide an identification. This often happened with non-fermenting gram-negative bacilli. Staphylococci were identified by coagulase reaction, thermostable nuclease, or by other conventional biochemical tests. Viridans group streptococci and enterococci were identified using an automated identification system.

Data was analyzed by the Chi square test using the SPSS statistical software package. Differences between the experimental groups were assessed as significant if the probability was <0.05.

RESULTS

Retrospective Study. The retrospective evaluation of islets from our cryopreserved tissue bank was performed and aliquots from a total of forty-seven preparations were thawed, cultured, and subjected to routine microbiological culture. A total of 27% (thirteen out of forty-seven) of the preparations had positive microbiological cultures after thawing (Table VI-1). The contaminated preparations grew microorganisms including Enterobacter cloacae (n=4), gram-negative bacilli (n=9), gram-positive cocci (n=1), and Viridans group strep (n=6) (Table VI-1). Multiple contaminants were found in seven cases. It was not possible to ascertain the source of the contaminants since bacterial cultures were not taken during the

individual steps of islet isolation culture *in vitro* or during cryopreservation. A mock islet isolation procedure was performed and microbial culture samples were taken at multiple steps throughout the isolation procedure. Gram-negative (aerobic) bacteria (Pseudomonas species) were found to be introduced on a settle plate in a biological safety cabinet which houses the isolation apparatus and from the Ficoll solution used to purify the islets. Procedures in draping, preservation of air flow in the biological safety cabinet, cleansing of the cabinets, and sterilization of the Ficoll solution were revised before proceeding with the prospective study detailed in the section that follows.

Prospective Study. Seventy consecutive islet isolations were followed prospectively. Pancreases procured locally by our transplant team had a rate of positive cultures of 11% when the pancreatectomy was performed before the multiorgan procurement, and 9% when the pancreas was removed as part of the multiorgan procurement with *in situ* UW flush (Table VI-2). Pancreases received from collaborative transplant centres had an infection rate of 26% (p=ns using Chi square test). These pancreases had a cold storage interval of 6 to 20 hours during shipping. Therefore, our overall results indicate that approximately 19% of the pancreases (thirteen out of seventy pancreases) received at our islet isolation centre are microbiologically contaminated upon arrival (Table VI-2). These pancreases were infected with gram-negative (n=5), gram-positive (n=6), and yeast (n=2) microorganisms. When the pancreases with positive cultures from the transport media were processed, the frequency of the originally detected contaminants decreased progressively to 3% at the end of the islet isolation process (Table VI-3).

This represents a 97% reduction in the contamination rate during the processing of the pancreas. The isolated islets were then cryopreserved and added to our low temperature tissue bank. None of these preparations which had positive cultures during the initial sampling of the transport media were found to acquire new contaminants during the sampling steps 6, 7, or 8 during the cryopreservation and thawing of the tissue (Table VI-3).

New contaminants identified during islet cell processing are described in Table VI-4. All pancreases in this group had no contaminants identified in the transport media. Our results indicate that a overall low incidence of microbial infection with exception of group 2, following pancreas perfusion. At this step approximately 12% of the pancreases showed evidence for microbial contamination. New contaminants were also introduced to the frozen-thawed islets during the sucrose dilution and persisted during the post-isolation culture period in two cases out of the twenty-seven preparations that were cryopreserved (7%). These preparations were removed from the tissue bank thereby precluding their use for transplantation.

To further evaluate whether our cryopreservation tubes became contaminated during the freeze-thaw process or during low temperature storage, two additional tubes containing sterile media only were cryopreserved at the same time that the preparation of islets was being frozen (n=6 cases). These tubes were immersed in the liquid phase of the liquid nitrogen solution together with their islet-containing counterparts. The first of these blank tubes was thawed at the same time that the tube containing islets was thawed for islet function and microbiological assessment. The second blank tube was thawed after approximately 3 months of being immersed

in liquid nitrogen with the islet preparation. There was no evidence for contamination in the blank tubes following storage short-term or prolonged (3 months) storage while immersed in liquid nitrogen.

DISCUSSION

After observing two patients who developed Enterobacter cloacae bacteremia following transplantation of freshly-isolated and frozen-thawed islets of Langerhans (8), we have investigated the frequency of microbial contamination in islets cryopreserved in our low temperature tissue bank. From a total of forty-seven cryopreserved islet preparations, aliquots from each preparation were randomly selected and thawed. Microbiological samples were removed immediately after thawing. In 27.6% of the preparations, contaminants were identified. Isolations with contaminated islets were immediately removed and discarded. As it had not been our practice to assay the steps in the isolation process we were unable to determine if the islets were contaminated before islet cryopreservation. A review of our aseptic techniques was then performed with assistance from the Department of Medical Microbiology & Infectious Diseases.

These results from our cryopreserved tissue bank demonstrate that any microorganisms present following islet isolation could persist and survive throughout the freezing and thawing process. The next seventy consecutive human islet isolations were vigorously sampled to identify the frequency of microbial contamination at multiple steps in the isolation, *in vitro* tissue culture, and cryopreservation procedures to identify any breaks in aseptic techniques.

Our method for collection of samples for microbial surveillance involves aseptically removing a 2 to 5 mL sample. Other islet transplant programs have collected specimens for microbiological analysis by filtering a 10 mL aliquot of the transport media through a 0.22 μm cellulose acetate filter (5), then the filter paper is subjected to microbiological analysis. The other method of obtaining samples for microbiological evaluation used by islet transplant programs is by introducing a small portion of liquid into culture vials. These vials contain either soybean-casein digest broth for anaerobic culture or tryptic soy broth for aerobic cultures. Anaerobic cultures were not taken. Although anaerobic microorganisms are possible, the entire process is completed under conditions exposed to atmospheric air which likely results in demise of anaerobes. The yield of positive anaerobic cultures would likely be small if present at all and not cost effective. No reports have previously identified anaerobic contaminants.

From the seventy pancreases prospectively studied, the number of pancreases contaminated initially upon arrival at our isolation centre during our study period was considerably lower than the 53% of positive cultures reported from pancreases received by the St. Louis islet isolation centre who receive pancreases from various transplant centres in the United States (5). The percentage of positive cultures from the UW transport media reported from the human islet transplant program in Pittsburgh was between 31 and 50% (6, 8). In both of these studies, the microbial contaminants were of gram-positive origin whereas gram-negative species were primarily observed during our study period. The etiology of these contaminants was not clear. We did not demonstrate a difference if pancreas procurement was initial

or after *in situ* flush. In our series, there was a trend towards an increased occurrence of infection with the procurement of pancreases by other procurement groups. This may have reflected differences in surgical technique or the more prolonged storage interval before islet isolation. Our finding that new contaminants were identified in up to 12% after initial pancreas perfusion is a new observation (Table VI-4). This may reflect inability to detect positive cultures in transport medium because some transport media is supplemented with penicillin. Steps have been taken to replace all silastic tubing and more stringent cleaning of the perfusion apparatus to prevent any further contamination.

Previous studies on microbial incidence during islet isolation suggest that the microbial contaminants are washed or diluted out during islet processing (5, 6). These observations are confirmed and further enlightened by our experimental results which show that the frequency of contamination decreases from 19% upon arrival, to 8% following digestion, to 3% following purification, to 0% following cryopreservation. Thus, the presence of contamination after collection of impure pancreatic fragments for clinical transplantation might be expected to yield higher risks of infusing contaminated preparations into recipients. The new contaminants that were introduced to the preparation occurred after the thawing process during the removal of the cryoprotectant agent. These incidents of contamination can be attributed to breaks in sterile technique during solution preparation.

The ability of glass freezer tubes containing only sterile tissue culture media to remain sterile after prolonged storage in liquid nitrogen is an important observation, and indicates that although the liquid nitrogen seeps into the tubes, the

contents in the tubes remain sterile upon thawing. Liquid nitrogen leaking into the glass tubes also creates potential hazards when the glass tubes are thawed, requiring the tubes to be opened and the liquid nitrogen poured off. We are in the process of developing a more efficient and safer method to cryopreserve large batches of isolated islets (19). This new system utilizes a large freezer bag which allows for easy addition and removal of the cryoprotectant agent minimizing the chance of microbial contamination and preventing the seepage of liquid nitrogen into the bag during prolonged storage.

Sampling for microbiological contamination at various steps in the isolation, *in vitro* culture period, and cryopreservation of islets allows for the evaluation of the sterility of the preparation and the identification of steps in the isolation that may introduce contaminants. Steps at which the culture become positive can be specifically identified and protocols modified to prevent any future contamination. These steps should be examined periodically in all islet transplant programs. We have found that a mock islet isolation procedure performed annually can help identify any breaks in sterile technique. This mock procedure includes the placement of settle plates for collection of airborne microorganisms in the laminar flow hoods. As well, all islets isolated and subsequently cryopreserved are quarantined until confirmation of the sterility of the preparation. Having islets in a cryopreserved state provides an opportunity for an aliquot of the preparation to be thawed and tested for both islet function and confirmation of sterility, while safely maintaining the remaining tissue for subsequent islet transplantation. In addition to sampling an aliquot of islets, following batch thawing of the preparation of islets for clinical

transplantation, a sample for microbiological culture is taken to confirm the sterility of the cryopreserved islets.

While the advent of disposable equipment will make all of the intermediate steps unnecessary, it is suggested that samples be removed from the transport media (Step 1), post-digestion (8 to 12% new contamination rate), post-isolation (Step 5), and following cryopreservation (Step 8) still be sampled for microbiological contamination. Sampling at these steps will allow sufficient monitoring of both the islet isolation and the cryopreservation process.

CONCLUSION

Our prospective study for monitoring microbiological contamination during the isolation and cryopreservation of islets indicate that the majority of the contamination is introduced with the donor pancreas in the transport media. The microorganisms can persist throughout the isolation, however, there is a reduction in the frequency of contamination during the islet processing. Contaminated islets which were cryopreserved remain contaminated upon thawing, however, there is no spreading of the contaminants into previously clean freezing tubes during the storage interval in liquid nitrogen.

We conclude that clinical islet transplant programs should perform microbial surveillance and documentation at multiple steps during the isolation and cryopreservation of the islets in order to identify the steps where contamination is introduced and in order to prevent the infusion of infected islets into immunosuppressed patients.

Table VI-1:

Retrospective evaluation of cryopreserved human islets (n=47) from a low temperature (-196°C) tissue bank.

Microorganisms	n	Percent
No contaminants	34/47	72.3
Enterbacter clocae	4	8.5
Gram -ve Bacilli	9	21.3
Gram +ve Cocci	1	2.1
Viridans group strep	6	12.8

Total 27.6% of islet preparations infected

7 islet isolations with multiple contaminants

Table VI-2:

Transport media infection rate according to the procurement method.

Group	Procurement Protocol	Program	n	# Infected	% Infected
1	Initial pancreatectomy	home	9	1	11
2	Pancreas post *in situ* flush	home	23	2	9
3	Pancreas post *in situ* flush	outside	38	10	26[a]
		TOTALS	70	13	19

[a] p Group 3 versus Group 2 = ns

Table VI-3:

Microbiological surveillance of islets isolated from pancreases received with infected transport fluid.

Protocol Step	Gram +	Gram -	Yeast	Number	%[a]
1. Transport medium	5	6	2	13/70	19
2. Perfusion	3	4	0	7	10
3. Digestion/dissociation	3	3	0	6	9
4. Post-purification	1	1	0	2	3
5. Pre-cryopreservation	0	0	0	0	0
6. Post-cryopreservation	0	0	0	0	0
7. Post-sucrose dilution	0	0	0	0	0
8. Post-cryo culture	0	0	0	0	0

[a] % of those originally infected

Table VI-4:

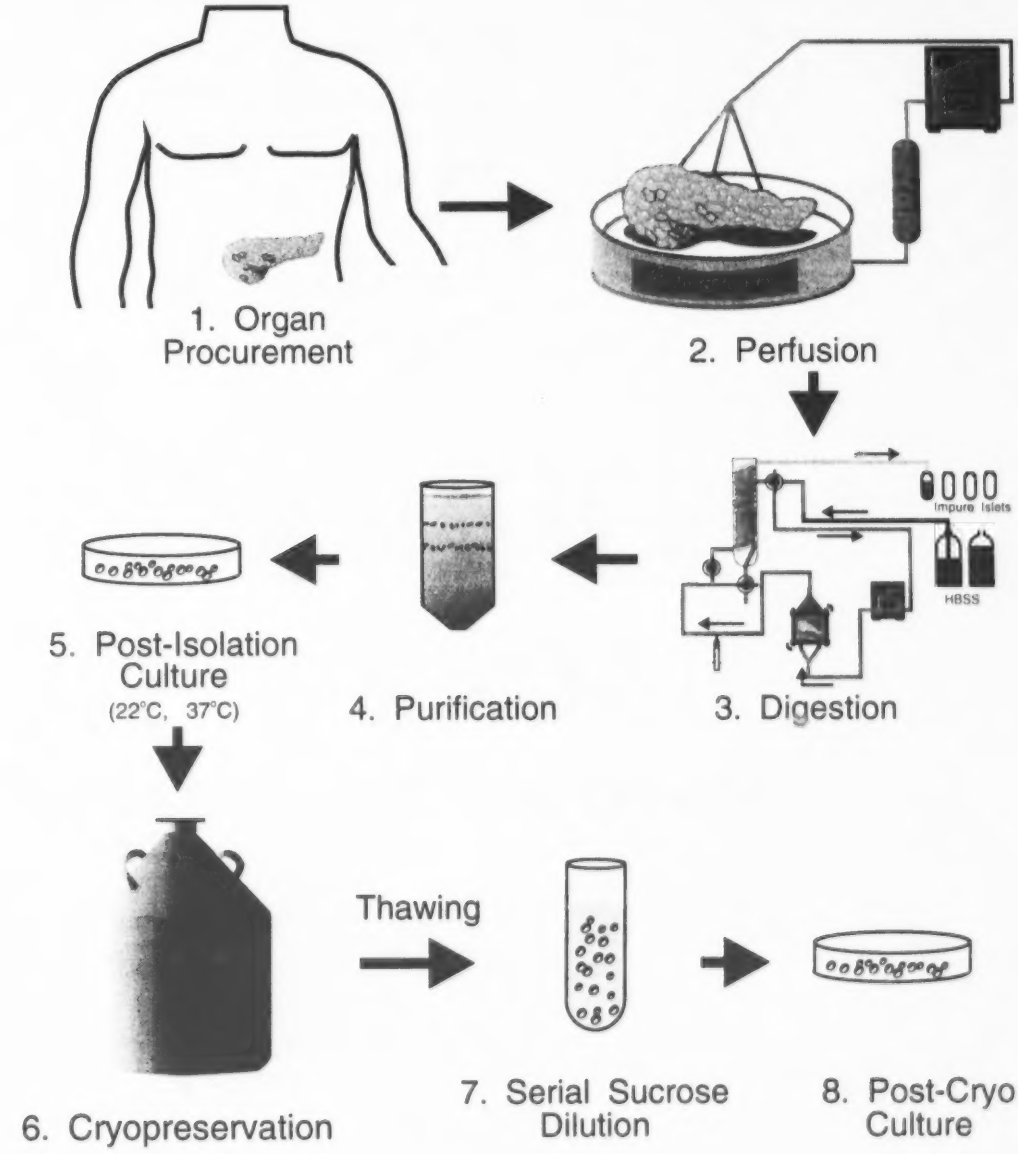
Prospective evaluation of new contaminants identified from pancreases with negative cultures in the transport media.

Protocol Step	Gram +	Gram -	Yeast	Number	%
1. Transport medium	0	0	0	0/57	0
2. Perfusion	1	3	2	6	12
3. Digestion/dissociation	1	1	2	4	8
4. Post-purification	0	1	0	1	2
5. Pre-cryopreservation	0	0	0	0	0
6. Post-cryopreservation[a]	0	0	0	0	0
7. Post-sucrose dilution[a]	0	2	0	2	7
8. Post-cryo culture[a]	0	2	0	2	7

[a] n=27

Figure VI-1:

Schematic illustrating experimental design for prospective study of microbiological sampling following each step: 1. organ procurement and transport; 2. pancreas perfusion; 3. digestion and dissociation of pancreas; 4. purification; 5. *in vitro* islet culture; 6. cryopreservation; 7. thawing and removal of cryoprotectant; 8. 48 hour *in vitro* culture.



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VII

BULK CRYOPRESERVATION OF ISOLATED ISLETS OF LANGERHANS[1]

INTRODUCTION

Transplantation of isolated islets has been shown to induce long-term normoglycemia and insulin independence in type 1 insulin-dependent diabetic patients (1-8). Evaluation of recent clinical islet transplants by the International Islet Transplant Registry identified several common characteristics in all patients achieving insulin independence following islet transplantation. One of these factors was the transplantation of an islet mass exceeding 6000 IE (islet equivalent) per kilogram body weight (5). Limitations in the ability to isolate such large numbers of viable islets make it necessary to pool islets from several donors to achieve this critical islet mass. Cryopreservation is an effective means of longterm storage of donor tissue. Additional benefits of islet cryopreservation include the creation of a low temperature tissue bank affording the opportunity to supplement freshly-isolated islets with cryopreserved islets in order to increase islet mass before transplantation (6). Of the cases reported to the International Islet Transplant Registry, type 1 diabetic recipients transplanted with freshly-isolated islets in combination with cryopreserved islets are insulin independent at 1 year in 18% of the cases (5). This is slightly higher than the 11% of the cases that are insulin independent when only freshly-isolated islets were transplanted (5). The type 1 diabetic patient with the longest duration of insulin independence to date, following islet transplantation

[1] A version of this chapter has been submitted for publication. Lakey JRT, Warnock GL, Ao Z, Rajotte RV. 1995. Cell Transplantation.

simultaneous to a renal transplant, received a combination of fresh and cryopreserved islets (5).

The current islet cryopreservation protocols are based on the protocols originally developed for the cryopreservation of rodent islets (9-11). These protocols use multiple glass freezing tubes and aliquot small numbers of islets per individual glass freezing tube. When these protocols are used for the cryopreservation of canine and human islets, the number of islets per glass tube is increased to 10-15,000 in a volume of 4 mL per glass tube. One human islet harvest can necessitate 30 to 40 glass tubes per freeze (11). Also, the freeze protocols using multiple tubes is labor intensive with the potential for microbial contamination. In addition, seepage of liquid nitrogen into the glass tubes during storage in liquid nitrogen is potentially hazardous during thawing in that the glass tubes may explode if they are thawed without first expelling the liquid nitrogen. As the number of clinical trials increase in various centres worldwide, there is a need to develop a more effective method for the cryopreservation of human islets. It was the aim of this project to develop and test a new method of bulk cryopreservation of isolated islets using a single freezer bag system which is less labor intensive, safe and minimize the risk of microbial contamination.

MATERIALS AND METHODS

Our initial evaluation of a freezer bag for the cryopreservation of large quantities of isolated islets involved a series of experiments to determine the actual pattern of heat transfer during the cooling and thawing process. A commercially

available freezer bag (500 mL capacity, Cryocyte freezer bag; Baxter Healthcare Corporation, Deerfield, IL) was fitted with Type T thermocouples (Cole Parmer; Niles, IL). The first thermocouple was placed in the upper one-third of the freezer bag, the second thermocouple in the middle, and the third thermocouple approximately one-third from the bottom. A fourth thermocouple was secured to the outside of the freezer bag as a reference for comparisons.

The freezer bag was filled with either 100 or 200 mL of 2 M DMSO (D128, Fisher Scientific; Ottawa, ON). The DMSO solution was volumetrically made up in freeze media, composed of Medium 199 containing 25 mM HEPES that had been supplemented with 10% v/v fetal calf serum (FCS) (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (P/S) (John's Scientific; Toronto, ON). The freezer bag was subjected to cooling and thawing protocols as used in the cryopreservation of islets (10, 11). Temperature profiles were generated from the thermocouples placed within the freezer bags. Temperatures were recorded every minute on a 2190A Fluke digital thermometer (Fluke; Mississauga, ON) for the duration of the experiment. Following this initial series of five runs, in order to achieve uniform temperatures throughout the bag, it was determined that a holder was necessary to maintain a uniform width across the freezer bag. A stainless steel holder was fabricated and placed around the freezer bag to ensure a uniform liquid thickness of 5 mm. The freezer bag was filled with 100 mL of 2 M DMSO solution for the second set of experiments with the stainless steel holder in place.

In order to compare standard glass tubes with the temperature profiles of the freezer bag, a 15 mL Kimax glass tube was fitted with one thermocouple

approximately 1 cm from the bottom of the tube. A second thermocouple was secured to the outside of the glass tube as a reference. The glass tube was filled with 4 mL of 2 M DMSO solution and was subjected to cooling and thawing protocols.

The next series of experiments were performed to determine the recovery of islets following the addition and removal of the cryoprotectant in a single freezer bag and multiple glass tubes without freezing the islets. Wistar-Furth rat islets were isolated using the techniques described by Lacy et al. (12). Known numbers of freshly-isolated rat islets were distributed into either a single freezer bag or multiple glass tubes. The DMSO in freeze media was added stepwise to minimize the osmotic stress to the islets (11). Briefly, islets were equilibrated with 0.67 M DMSO for 5 minutes, then with 1 M DMSO for 30 minutes. The concentration of DMSO was then increased to 2 M and 15 minutes was allowed for equilibration to occur (11). The freezer bag and glass tubes were then cooled to 4°C in an ice slush for 5 minutes and then transferred to a -7.4°C ethanol seeding bath for 15 minutes. At this point, the islets were removed from the ethanol bath and the DMSO was removed at 4°C using a sucrose dilution (10, 11). The contents of the bag were drained into two 50 mL Falcon tubes, centrifuged, and the supernatant removed. The glass tubes were centrifuged and the supernatant removed. Five milliliters of 4°C 0.75 M sucrose was added to each of the 50 mL tubes which held the contents of the freezer bag. One milliliter sucrose solution was added to the glass tubes. After 30 minutes at 4°C, the sucrose was diluted using a step dilution (11). Medium 199 (Gibco) containing 25 mM HEPES, 10% FCS and P/S was serially added in 5 minute intervals after which the tubes were centrifuged, the supernatant removed, and the islets transferred

to an isotonic medium. To assess islet recovery, the total number of islets recovered were recounted with the aid of a dissecting microscope that had been fitted with a green filter (13). The total number of islets recovered was compared with the precryopreservation numbers and the percent recovery calculated.

In order to determine the recovery and function of islets following cryopreservation in the freezer bag, we performed a series of experiments in which freshly-isolated canine islets were cryopreserved in a single freezer bag and compared with islets cryopreserved in multiple glass tubes. Islets were isolated from pancreases of outbred mongrel dogs using collagenase digestion and Ficoll purification as previously described (14-17). Duplicate aliquots of tissue were removed, stained with dithizone (18), and the islet mass was quantified by two independent investigators in accordance with the criteria established at the 1989 International Workshop on Islet Assessment (19). The number of islets in each diameter class was calculated by counting and sizing each islet under a dissecting microscope that had been fitted with an optical graticule. The number of islets was then converted to the standard number of IE (number of islets of 150 μm diameter equal in volume to sample). The mean of the two counts was calculated and compared with the precryopreservation numbers and the percentage recovery was determined.

Aliquots of freshly-isolated canine islets were suspended in the freeze media and then allocated into a single 500 mL capacity Cryocyte freezer bag or multiple 15 mL Kimax glass freezer tubes. DMSO was added stepwise to a final concentration of 2 M (11). Following the final equilibration, the samples were transferred to an ice slush bath for 5 minutes, then to a -7.4°C 95% ethanol seeding bath for 10

minutes before nucleation. Nucleation was achieved by placing a supercooled metal rod against the side of the freezer bag and tubes. Gentle mixing allowed the ice crystals to propagate throughout the freezer bag and the glass tubes. After allowing time for the release of the latent heat of fusion, the islets were slowly cooled at 0.25°C/minute to -40°C using a programmable FTS freezer bath system (FTS Inc.; Stone Ridge, NY). At -40°C the stainless steel holder was removed from the bag and both the bag and tubes were plunged into liquid nitrogen (-196°C) for low temperature storage. Following a period of storage, the bag and the tubes containing cryopreserved canine islets were thawed (175-200°C/minute) by rapid agitation in a 40°C water bath to 0°C and placed in a ice slush bath. The contents of the bag were drained into two 50 mL Falcon tubes, centrifuged, and the supernatant removed. The glass tubes were centrifuged and the supernatant removed. Five milliliters of 4°C 0.75 M sucrose was added to each of the 50 mL tubes which held the contents of the freezer bag. One milliliter sucrose solution was added to the glass tubes. After 30 minutes at 4°C, the sucrose was diluted using a step dilution (11). Duplicate aliquots of the islet tissue from both experimental groups were removed, stained with dithizone, counted and sized (19). The percent islet recovery was calculated by comparing pre- and post-cryopreservation counts for each group.

Following cryopreservation and before viability testing, the frozen-thawed canine islets were cultured in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C for a period of 48 hours in CMRL 1066 tissue culture media (Gibco) which had been supplemented with 10% FCS, 25 mM HEPES, and P/S. Known numbers of canine islets were placed in modified Millipore chambers (20) and perifused with

Krebs (Gibco) solution containing glucose in the following concentrations: initially 2.8 mmol/L for 60 minutes, then 28 mmol/L for 60 minutes, and finally 2.8 mmol/L for the remaining 60 minutes. Samples for insulin determination were collected and the insulin concentration of the perifusate was determined using double antibody radioimmunoassay with commercially available RIA kits (Pharmacia; Uppsala, Sweden) and human insulin standards (21). The stimulation index was determined from the insulin values during glucose perifusion and represents the mean insulin secretion of the islets during the high glucose (28 mmol/L) stimulation period over the mean insulin secretion during both periods of low basal insulin secretion (pre- and post-stimulation) (22, 23).

For *in vivo* viability assessment 2000 frozen-thawed canine islets (IE) cryopreserved in the freezer bag or from the glass tubes were placed under the kidney capsule of Balb/c nude mice (Banting Laboratories; Seattle, WA) that were rendered diabetic by a single intravenous injection of alloxan (90 mg/kg) (ICN Biomedicals; Costa Mesa, CA) (24, 25). Only mice with non-fasting blood glucose levels above 20 mmol/L were transplanted. Tail vein blood glucose samples were taken twice a week and measured using a Companion 2 blood glucose meter (Medisense; Waltham, MA). For comparative purposes, freshly-isolated canine islets were transplanted into diabetic nude mice.

Statistical Analysis. Results are expressed as mean ± standard error of the mean (mean ± SEM). Differences between the groups were assessed using the Mann Whitney U test using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

The freezing and thawing profiles generated by placing thermocouples into a freezer bag containing 100 or 200 mL of 2 M DMSO solution and held at a uniform width of 5 mm using the metal holder are illustrated in Figures VII-1A through C. Figure VII-1A shows that during the initial cooling of five individual experiments with a freezer bag filled with 100 mL of 2 M DMSO solution, there was a delay in the temperature response within the freezer bag when the bag was cooled from room temperature to 0°C (ice slush solution). The temperature, however, within all regions of the bag reached the ice slush temperature before the bag was transferred to the -7.4°C ethanol seeding bath. A temperature delay was also observed when the freezer bag was placed in the -7.4°C ethanol bath. This nonuniform temperature within the bag may account for the difficulties experienced when attempting to nucleate the freezer bag after 5 minutes in the ethanol bath. A slow response to cooling was also exhibited in the series of five experiments when the bag was filled with 200 mL of solution (Figure VII-1B). We were unable to nucleate the freezer bag after only 5 minutes in the ethanol bath. To ensure uniform supercooling throughout the bag the time before nucleation at -7.4°C was increased to 10 minutes (Figure VII-1C). Figure VII-1C shows that by 10 minutes the temperatures in all regions of the bag had reached -7.4°C.

The cooling profiles of the glass tubes fitted with the thermocouples were similar to those obtained for the freezer bag. Temperature delays were also evident following placement into the ice slush and ethanol seeding bath, however, the

temperature reached -7.4°C within 4 minutes following placement in the ethanol bath (Figure VII-2).

When the freezer bag was slowly cooled at 0.25°C/minute to -40°C the cooling rates from -7.4°C to -40°C were uniform in all regions of the freezer bag (Figure VII-3). The temperature profiles during rapid thawing of the freezer bag are shown in Figure VII-4. The measured thawing rates from -196°C to 0°C were approximately 175-200°C/minute for both the freezer bag and the glass tubes.

To determine the recovery of islets following the addition and removal of the cryoprotectant in the freezer bags without freezing, groups of 500 freshly-isolated Wistar-Furth rat islets from four individual experiments were placed in either a freezer bag or multiple glass tubes. Islet recovery from the freezer bag was 92±4.8% (mean±SEM) and 90±2.3% from the glass tubes (n=4) (p=ns, Mann Whitney U test) (Table VII-1).

To assess the recovery and function of frozen-thawed canine islets groups of 10,000 to 35,000 IE were cryopreserved in either a single freezer bag or in multiple glass tubes. Following rapid thawing and sucrose dilution, 78.7±12.5% of the islets cryopreserved in a single freezer bag were recovered vs 82.3±5.3% in multiple glass tubes (n=6 individual experiments, p=ns) (Table VII-2). After 48 hours of tissue culture at 37°C both groups of islets had a similar basal and stimulated response to glucose during perifusion (Figure VII-5, Table VII-3). The mean insulin secretion from islets frozen in the freezer bag rose from a baseline of 0.027±0.012 μU insulin/IE/minute during the low glucose phase to a mean insulin secretion of 0.066±0.014 during the high glucose stimulation phase. Whereas, islets frozen in

glass tubes rose from a mean insulin secretion of 0.032 ± 0.006 μU insulin/IE/minute to 0.057 ± 0.009 (p = ns) (Table VII-3). Non-frozen canine islet controls cultured for 48 hours at 37°C before glucose perifusion had a 7-fold increase in mean insulin secretion from 0.014 ± 0.01 to 0.09 ± 0.01 μU/islet/minute during the high glucose stimulatory phase. Insulin secretion in all groups returned to basal levels when the islets were returned to the low glucose solution. The stimulation index which is calculated by dividing the amount of insulin secreted during the high glucose stimulation phase divided by the insulin secretion during both low glucose phases of the perifusion was 3.2 ± 1.0 for the islets cryopreserved in the freezer bag and 2.3 ± 1.3 for islets cryopreserved in the glass tubes (p = ns). These values are significantly lower than the calculated stimulation index for fresh non-frozen controls (6.9 ± 2.4) ($p < 0.05$), which is commonly seen when assessing *in vitro* function of cryopreserved islets. The stimulation index for the bag and the tubes, however, were not significantly different from each other (p = ns).

The *in vivo* assessment of the frozen-thawed canine islets transplanted into alloxan-induced diabetic nude mice is shown in Table VII-4. Five of the eight diabetic nude mice transplanted with 2000 frozen-thawed islets cryopreserved in a freezer bag maintained normoglycemia for >30 days as compared with four of the six receiving islets cryopreserved in glass tubes (p = ns) (Figure VII-6). One animal from each group failed to return to normoglycemia. Five of six nude mice receiving 2000 freshly-isolated canine islets became normoglycemia long-term (Table VII-4). In comparing the cryopreservation groups, we observed no difference in the time to return to normoglycemia with all mice having blood glucose values below 10 mmol/L

within 1 week post-transplant (Figure VII-6). Removal of the kidney bearing the islet graft at 30 days post-transplant resulted in prompt hyperglycemia in all groups of nude mice.

DISCUSSION

Cryopreservation of isolated islets is one method of storing and pooling tissue before transplantation. Cryopreserved human islets have been used to supplement freshly-isolated islets above 6000 IE per kilogram body weight which seems to be needed for patients with longstanding diabetes. Several patients receiving both fresh and cryopreserved islets have experienced periods of insulin independence following transplantation (1, 5, 26). Transplantation of cryopreserved islets alone has been attempted, however, to date no recipients have become insulin independent (5, 27).

The ability to safely store islets in a low temperature tissue bank allows the frozen tissue to be shipped from one centre to another with ease (11). Low temperature banking also enables specific HLA tissue matching (11) and permits the induction of donor specific allograft unresponsiveness (28).

In 1977 Rajotte et al. (9) and Ferguson et al. (29) were the first to successfully restore euglycemia long-term following transplantation of frozen-thawed islets into streptozotocin-induced diabetic rats. Since then, a number of islet cryopreservation protocols have been reported (9, 29-32). In 1989, Rajotte et al. compared a variety of reported cryopreservation protocols and found that slow cooling to -40°C in combination with rapid thawing resulted in similar *in vitro* function comparable to the non-frozen controls (32). Current practices of human islet cryopreservation follow

those originally developed for the rodents which involved allocating islets to multiple glass freezer tubes (9). This is both labor intensive and, when dealing with a large number of tubes from a typical islet isolation, has an increased possibility for microbial contamination. The potential for seepage of liquid nitrogen into the glass tubes during storage increasing the risk of contamination as well as the potential of the glass tubes exploding if the liquid nitrogen is not removed before thawing.

There are several commercially available blood freezer bags designed for long-term storage of blood and blood products. These bags are composed of a thermoresistant plastic that is both nontoxic and able to resist the mechanical stresses at extremely low temperatures. Lymphocytes, bone marrow, and peripheral blood stem cells have been effectively cryopreserved in these large capacity freezer bags (33, 34). However, there have been no reports on the feasibility of the cryopreservation of islets in such bulk freezer bags. It was the aim of this project to develop and test a new and more efficient method of human islet cryopreservation using a commercially available blood freezer bag.

Initial evaluation of a commercially available freezer bag, with thermocouples placed in specific regions of the freezer bag, demonstrated that during the cooling and thawing processes heat transfer throughout the bag was not uniform unless the freezer bag was placed in a stainless steel holder and sufficient time was allowed for supercooling before nucleation.

When the percentage recovery was assessed during and after the addition of the cryoprotectant without freezing, there was a 10% loss of islets; which was equivalent to that seen in the glass tubes. These losses are most likely due to islets

adhering to the sides of the containers and the losses associated with the removal of the cryoprotectant and tissue recombination.

When freshly-isolated, highly-purified canine islets were cryopreserved in the freezer bag, the percentage recovery and *in vitro* viability was comparable to islets cryopreserved in the glass tubes. Transplantation of frozen-thawed canine islets cryopreserved in either a freezer bag or in glass tubes were able to reverse and normalize the blood glucose in alloxan-induced diabetic nude mice.

The ability to bulk freeze islets in a single bag offers several advantages over multiple glass tubes. The entire preparation of islets can be frozen in one bag, which is simpler and less prone to inadvertent mixing of tubes. Cryopreservation in a single freezer bag is more cost effective as it is less labor intensive and requires less dewar storage space. The freezer bag system has now been developed to allow for the automated addition and removal of the cryoprotectant, adding consistency and reproducibility to the cryopreservation process.

CONCLUSION

Cryopreservation of isolated islets in a single freezer bag allows for equally effective storage while facilitating the automated addition and removal of the cryoprotectant agent while minimizing the chance of microbial contamination. This series of experiments will assist in the development of a more effective and automated method to cryopreserve human islets, thus allowing for the establishment of a more effective low temperature tissue bank to support ongoing clinical islet transplant trials.

Table VII-1:

Recovery of isolated rat islets following stepwise addition of DMSO to a final concentration of 2 M, supercooling to -7.4°C and removal of the cryoprotectant using a 0.75 M sucrose dilution.

Group	n	Percent Recovery (mean ± SEM)
Glass tube	4	88, 95, 85, 93 (90 ± 2.3)
Freezer bag	4	101, 79, 91, 97 (92 ± 4.8)

Table VII-2:

Recovery of purified canine islets following cryopreservation in either a freezer bag or glass tubes.

Group	n	Percent Recovery (mean ± SEM)
Glass tube	6	78, 102, 88, 85, 63, 78 (82.3 ± 5.3)
Freezer bag	6	134, 49, 83, 55, 67, 83 (78.8 ± 12.5)

Table VII-3:

Calculated stimulation index from the glucose stimulated perifusion response of canine islets cryopreserved in either a freezer bag or in glass tubes.

Group	n	Insulin Secretion (μU/IE/minute)		Calculated Stimulation Index
		Basal	Stimulated	
Non-frozen controls	4	0.014 ± 0.01	0.09 ± 0.01	6.9 ± 2.4
Glass Tubes	6	0.032 ± 0.006	0.057 ± 0.009	2.3 ± 1.3
Freezer bag	6	0.027 ± 0.012	0.066 ± 0.015	3.2 ± 1.0

Table VII-4:

Survival and function of fresh and cryopreserved islet grafts transplanted under the kidney capsule of alloxan-induced diabetic Balb/c nude mice.

Group	n	Days of Normoglycemia[a]	Median	% Success at 30 days
Islets cryopreserved in freezer bag	8	0, 16, 22[b], >30x5	30	62.5
Islets cryopreserved in glass tubes	6	0, 16, >30x4	30	66.7
Freshly-isolated islets	6	0, >30x5	30	83
Non-transplanted controls	5	4, 6, 8, 8, 10	8	0

[a] Blood glucose < 10 mmol/L

[b] Died while normoglycemic

Figure VII-1:

Temperature profiles generated by placing thermocouples in a freezer bag. Probe positioned at upper one-third of bag (open triangle), positioned in middle of bag (closed circle), positioned at bottom one-third of bag (open triangle), and placed on outside of bag (closed square).

Freezer bag containing (A) 100 mL or (B) 200 mL of 2 M DMSO solution, supercooled for 5 minutes before attempting to nucleate or (C) freezer bag containing 100 mL of 2 M DMSO solution with cryopreservation protocol modified to allow 10 minutes of supercooling at -7.4 °C prior to nucleation.

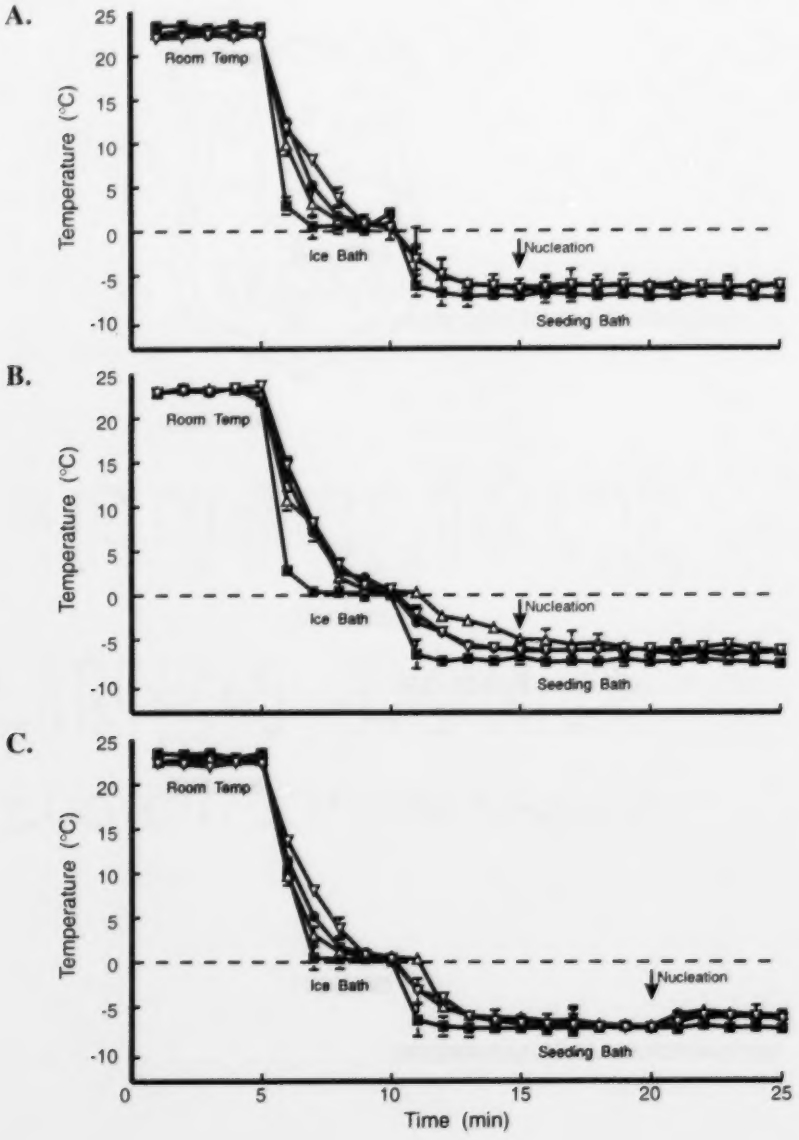


Figure VII-2:

Temperature profile generated with a single thermocouple placed within a single glass freezer tube (closed square) and subjected to the standard islet cryopreservation protocol. The second probe (open triangle) was placed on the outside of the tube as a reference.

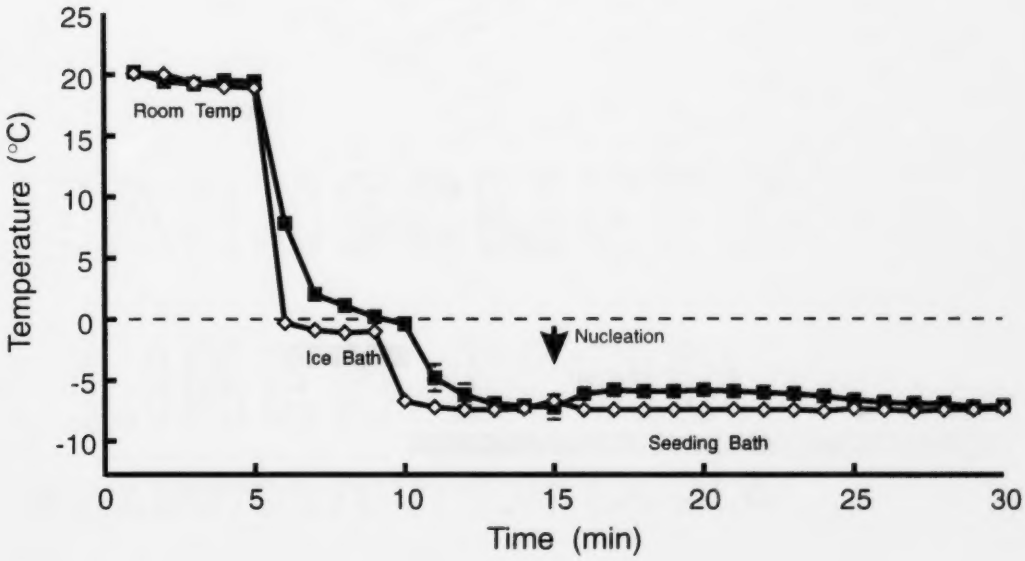


Figure VII-3:

Temperature profile from a freezer bag containing three thermocouples and 100 mL of 2 M DMSO solution during slow cooling at 0.25°C/minute from -10°C to -40°C. Probe positioned at upper one-third of bag (open triangle), positioned in middle of bag (closed circle), positioned at bottom one-third of bag (open triangle), and placed on outside of bag (closed square).

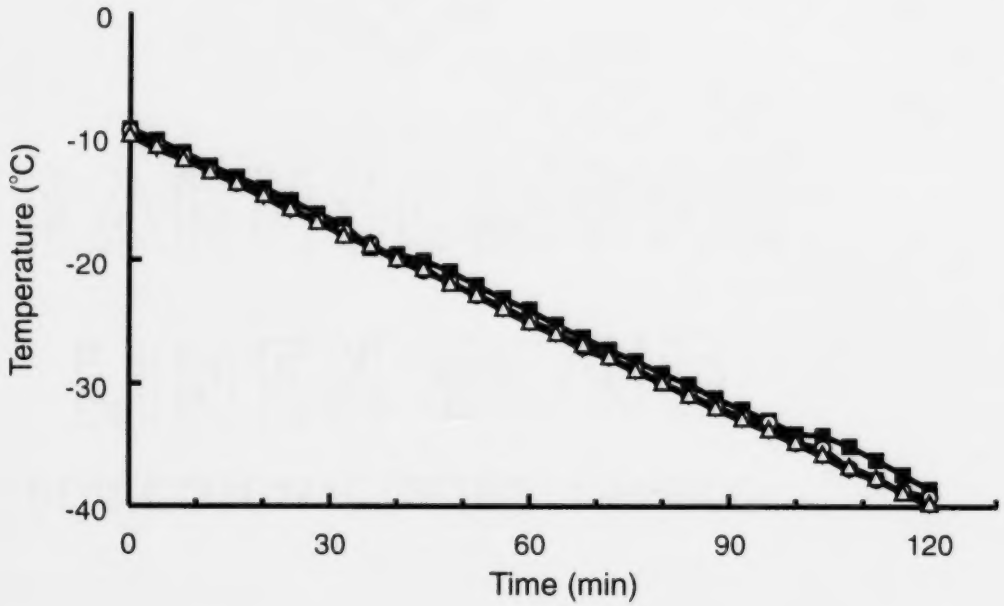


Figure VII-4:

Temperature profile from a freezer bag containing three thermocouples and 100 mL of 2 M DMSO solution during rapid thawing from -196°C to -0°Cin a 40°C water bath with a calculated thawing rate of 175-200°C/minute.. Probe positioned at upper one-third of bag (open triangle), positioned in middle of bag (open circle), positioned at bottom one-third of bag (closed square).

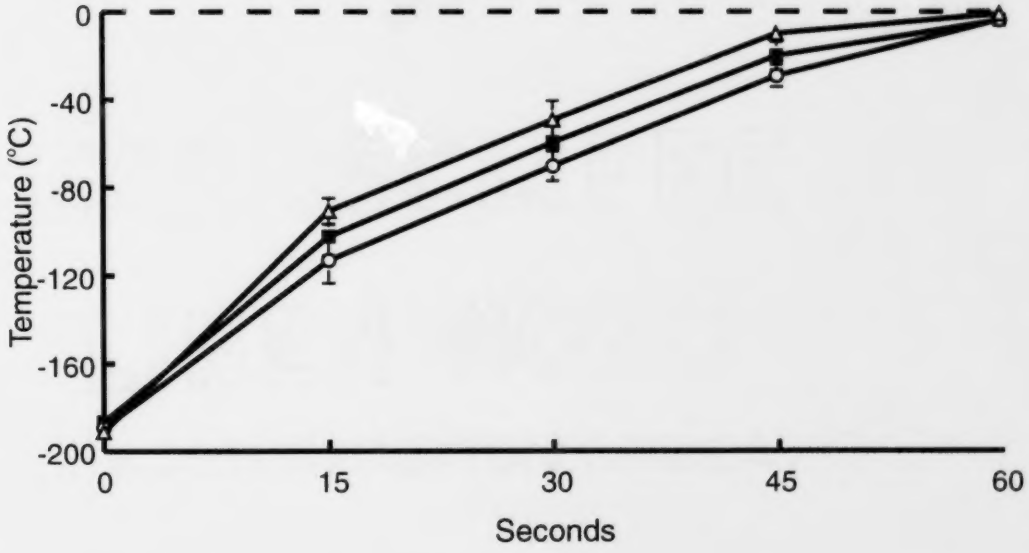


Figure VII-5:

Glucose stimulated perifusion response of canine islets either cryopreserved in a freezer bag (closed diamonds) or in glass tubes (open triangles). Non-frozen canine islets (closed squares) from the same islet isolation are included as controls.

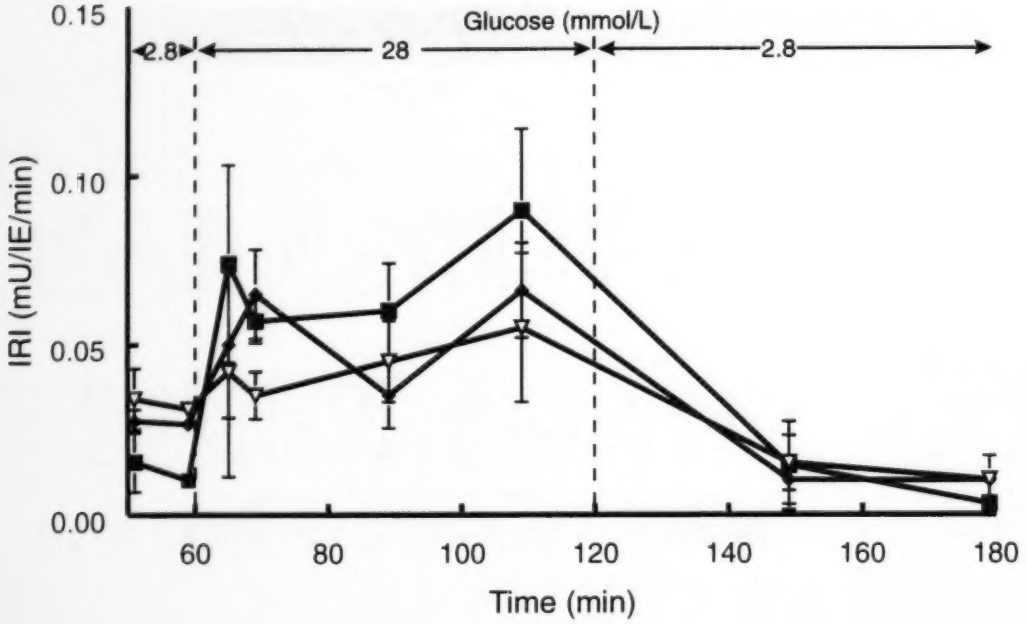
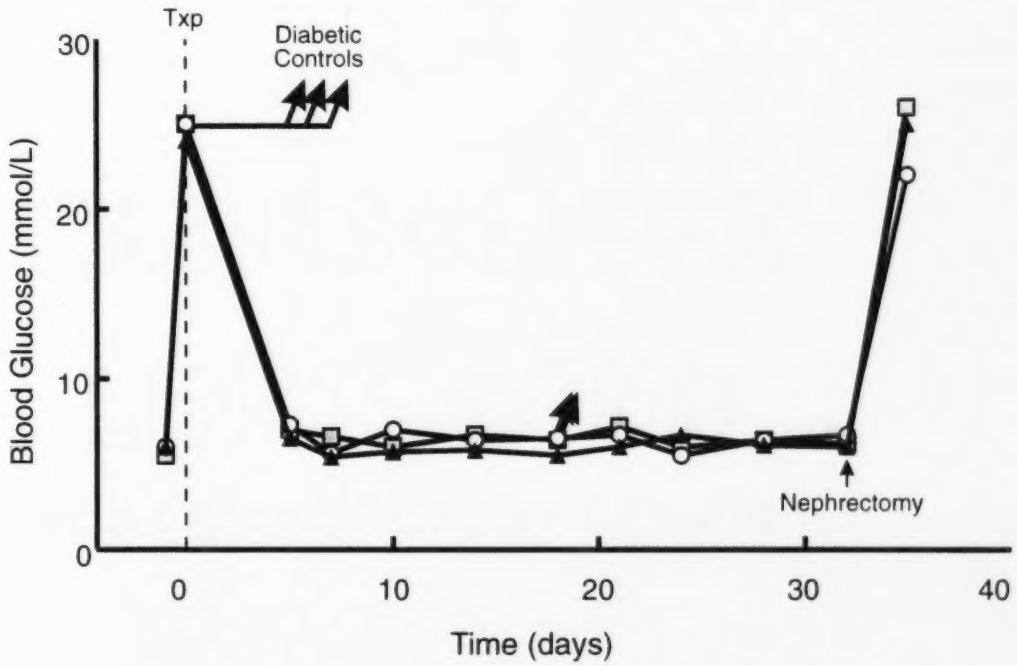


Figure VII-6:

Non-fasting blood glucose profiles of alloxan induced diabetic nude mice receiving 2000 frozen-thawed canine islets cryopreserved in a freezer bag (open circles) or glass tubes (shaded box). Non-frozen canine islets (closed diamonds) are included as controls for comparative purposes. At 30 days post-transplant removal of the islet graft resulted in prompt hyperglycemia. Arrow = failed transplant.



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VIII

COMPARISON OF METHODS USED FOR THE REMOVAL OF DMSO FOLLOWING CRYOPRESERVATION AND THE DEVELOPMENT OF AN AUTOMATED PROTOCOL[1]

INTRODUCTION

Cryopreservation of isolated human islets is an effective method of longterm storage. Cryopreserved islets from low temperature tissue banks have been used to increase the transplantable islet mass before clinical islet transplantation in patients with longstanding insulin dependent diabetes mellitus (1-3). Several of these patients which have received islets from low temperature tissue banks have experienced periods of insulin independence following transplantation (2-5). In addition to safe low temperature preservation of tissue, the creation of a low temperature tissue bank with multiple HLA tissue types allows the selection of specific islet preparations based on HLA tissue matching and allows the evaluation of islet function and confirmation of microbial sterility before transplantation (1, 6, 7). Cryopreserved islets can be easily shipped between collaborative transplant centres (8) and effective low temperature storage permits the time required for the induction of donor specific unresponsiveness (9, 10). Recently we have developed a method for the bulk cryopreservation of islets in a single blood freezer bag in an attempt to improve the methods used to bank islets (11, 12). This storage method has several advantages over the conventional methods of low temperature

[1] A version of this chapter has been submitted for publication. Lakey JRT, Warnock GL, Rajotte RV. 1995. Cell Transplantation.

storage in multiple glass tubes including the increased ease of banking (12). An additional advantage of cryopreservation in the freezer bag is the potential to develop an automated protocol for the addition and removal of the cryoprotectant.

The two approaches currently used to remove the cryoprotectant following thawing of islets are sucrose dilution or slow step dilution protocol. The sucrose dilution utilizes an exposure to a 0.75 M hyperosmotic sucrose solution following centrifugation and removal of the supernatant (1, 13). The sucrose is then removed by a slow serial dilution. The second approach is the slow step dilution protocol in which the cryoprotectant is slowly removed by a stepwise addition of an isotonic solution (14, 15). It was the aim of this study to develop an automated protocol which allowed both the addition and the removal of the cryoprotectant within the freezer bag system. The slow step protocol was not feasible because the total volume of dilution media exceeded the capacity of the freezer bag. Therefore, we adapted the slow step protocol to enable the entire process to be completed within the freezer bag.

MATERIALS AND METHODS

Islet Isolation, In vitro Culture and Quantification. Pancreatic islets were isolated from mongrel dogs using controlled perfusion via the duct, automated dissociation and discontinuous Ficoll purification as previously described (16-18). Groups of purified islets were placed in CMRL 1066 tissue culture solution (Gibco, Massasauga, ON) containing 10% fetal calf serum (FCS; Gibco), 25 mmol/L HEPES (Gibco) and 100 unit/mL Penicillin and 100 ug/mL Streptomycin (P/S; Gibco) and

were placed in tissue culture plates in a humidified atmosphere of 95% air and 5% CO_2. Following overnight tissue culture, islets were then recombined and duplicate aliquots of known volumes of tissue were stained in dithizone (Sigma Chemical Co., St. Louis, MO) (19, 20). The aliquot was then examined microscopically with the aid of an optical micrometer and the islet mass quantified by two independent observers in accordance to established criteria (21). The crude number of stained islets in each diameter class were counted and sized and converted to the standard.

Islet Freezing and Low Temperature Storage. In the first series of experiments, groups of canine islets (10-35,000 IE) were suspended in Medium 199 solution (Gibco) containing 10% FCS and P/S (Freeze Media). They were equally distributed into three groups of glass tubes and dimethyl sulfoxide (DMSO; Fisher, Toronto, ON) was added in a stepwise fashion to minimize the osmotic stress to the islets (1, 13). Islets were first equilibrated with 0.67 M DMSO for 5 minutes and then with 1 M DMSO for 30 minutes. The concentration of DMSO was then increased to 2 M and 15 minutes was allowed for the final equilibration (1). The total volume in the glass tubes was 4 mL. Following the final equilibration with 2 M DMSO solution, the glass tubes were capped and transferred to an ice slush bath for 5 minutes, then to a -7.4°C 95% ethanol seeding bath for 5 minutes before nucleation. Nucleation was achieved by placing a supercooled metal rod against the side of the glass tubes. Gentle mixing allowed the ice crystals to propagate throughout the glass tubes. After allowing time for the release of the latent heat of fusion, the islets were slowly cooled at 0.25°C/minute to -40°C using a programmable

FTS freezer bath system (FTS Inc.; Stone Ridge, NY). At -40°C the tubes were plunged into liquid nitrogen (-196°C) for low temperature storage (1).

In the second set of experiments islets were suspended in Freeze Media and allocated into 500 mL capacity Cryocyte freezer bags (Baxter Healthcare Corporation, Deerfield, IL). The DMSO was added using an automated protocol; adding the concentrations of DMSO at preprogrammed intervals to minimize the osmotic stress as described above (12). Following the final equilibration with 2 M DMSO solution, the freezer bags were heat sealed and transferred to an ice slush solution for 5 minutes. Bags were transferred to the ethanol seeding bath as described above and nucleated in the same fashion. The bags were then placed in a stainless steel holder in order to maintain a uniform width across all regions of the bag. Following sufficient time for the release of the latent heat of fusion, bags were slowly cooled at 0.25°C/minute to -40°C using the FTS freezer bath system. At -40°C the stainless steel holder was removed and the freezer bag was plunged into liquid nitrogen for low temperature storage.

Thawing and Removal of DMSO. In the initial set of experiments, following a period of storage in liquid nitrogen, the glass tubes containing cryopreserved canine islets were thawed rapidly (175-200°C/minute) to 0°C and the cryoprotectant was removed using the three dilution protocols (Figure VIII-1-3). In the first group (Group 1), frozen-thawed islets were centrifuged for 30 seconds at 1500 rpm (450 g), and the supernatant was removed (Figure VIII-1). The islets were then exposed to a 0.75 M sucrose solution for a period of 30 minutes at 0°C. The sucrose was then diluted with stepwise addition of Medium 199 solution (Gibco) that had been

supplemented with 10% FCS (Gibco), P/S and 25 mmol/L HEPES (Gibco) (Thaw Media) (1). The second method (Group 2) utilized a dilution protocol which involved a slow stepwise addition of Thaw Media to minimize the osmotic stress to the islets (14) (Figure VIII-2). This protocol was carried out at 22°C. One milliliter of room temperature Thaw Media was added to the tube containing the thawed islets every 2 minutes for the first 8 minutes. At 10 minutes 2 mL of Thaw Media was added every 2 minutes until the total volume in the tube was 16 mL. At this point, 4 mL aliquots were added four times at 2 minute intervals. The final 8 mL aliquot was added to the tube at 26 minutes. The final volume in the tube was 40 mL and the DMSO concentration has been reduced to 0.2 M.

The third protocol (Group 3) involved a modified slow stepwise addition of Thaw Media (Figure VIII-3). This protocol is similar to the slow step dilution protocol (Group 2), however, the total volume of Thaw Media that was added to the tube was reduced and the addition sequence was spread over a 30 minute time period. Every 5 minutes 2 mL aliquots of room temperature Thaw Media were added to the glass tubes until the overall concentration of DMSO in the tube was reduced to 0.5 M concentration.

In the second set of experiments the groups of islets cryopreserved in freezer bags were rapidly thawed in a 40°C waterbath to 0°C and the DMSO removed using either the standard sucrose dilution protocol (Group 4) or using the modified slow step protocol (Group 5). For islets in Group 4, the contents of the freezer bag were drained into two 50 mL Falcon tubes, centrifuged at 450 g for 30 seconds and the supernatant removed. Five milliliters of 0.75 M sucrose solution was added to each

tube and the tubes were kept in an ice slush bath for 30 minutes. The sucrose was then serially diluted with Thaw Media using previously described protocols (1).

Following thawing of islets from Group 5 the DMSO was removed using an automated protocol where 50 mL of Thaw Media was added to the freezer bag using a computer controlled system at 5 minute intervals until the concentration of DMSO in the freezer bag was 0.5 M. The time sequence used was based on diffusion chamber experiments which showed that islets return to an isotonic volume within 5 minutes when exposed to a hypotonic solution. The contents of the freezer bag (400 mL) was then transferred into two 250 mL conical tubes (Corning, Corning, NY) and centrifuged at 450 g for 30 seconds before the supernatant was removed. The frozen-thawed islets were then transferred to an isotonic media.

Following removal of the cryoprotectant, duplicate aliquots of the islet tissue from all groups were removed, stained with dithizone, and the islet mass quantified as previously described (21). The percent islet recovery was calculated by comparing pre and post-cryopreservation counts for each group. Following cryopreservation and before *in vitro* and *in vivo* viability testing, the frozen-thawed canine islets were cultured in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C for a period of 48 hours in CMRL 1066 tissue culture media (Gibco) which was supplemented with 10% FCS, 25 mmol/L HEPES, and P/S.

Static Incubation. To assess *in vitro* viability of the frozen-thawed islets from the various groups known numbers of islets were incubated in RPMI solution (Gibco) that had been supplemented with low (2.8 mmol/L), high (20 mmol/L) glucose or high glucose solution supplemented with 50 μm IBMX (Aldrich, Milwaukee, Wi) (22,

23). After 2 hour incubation at 37°C in a humidified 5% CO_2 incubator, the supernatant was assessed for insulin content using double antibody radioimmunoassay and human insulin standards (24). The insulin secretory capabilities of the islets were expressed as insulin per islet equivalent per hour.

In vivo Graft Assessment. *In vivo* evaluation of the frozen-thawed canine islets from both series of experiments were evaluated by transplantation of 2000 frozen-thawed islets under the kidney capsule of 5-7 week old Balb/C nude mice (Banting Laboratories, Seattle, WA) (24). Mice were housed under VAF conditions and were rendered diabetic by single intravenous injection of alloxan (90 mg/kg, ICN Biomedical, Costa Mesa, CA) (25). Only mice with nonfasting blood glucose values >20 mmol/L were used as transplant recipients.

Following islet transplantation blood glucose samples were drawn twice a week from tail vein blood samples using a Companion 2 blood glucose meter (Medisense, Waltham, MA). Grafts were considered successful if the plasma glucose returned to below 8.4 mmol/L.

Oral Glucose Tolerance Test. At 50 days post-transplant all euglycemic mice were tested for glucose tolerance. Three grams glucose/kg body weight (50% solution, Abbott Laboratories, Montreal, PQ) was given by oral gavage into unanaesthetized mice which were fasted for 2 hours. Blood samples were taken from tail vein at 0, 15, 30, 60 and 120 minutes. Non-transplanted age matched normoglycemic nude mice and mice that had received 2000 freshly isolated canine islets under the kidney capsule were used as controls for comparative purposes.

Histology. The kidney bearing the islet graft was removed and fixed in a 2% Bouins solution for 2 hours and then transferred to a 70% ethanol solution. Paraffin embedded grafts were then sectioned and stained with haematoxylin and eosin or Gormori's aldehyde-fuchsin. All histological sections were evaluated by a clinical pathologist. Following removal of the kidney bearing the islet graft, the mice were monitored until they became hyperglycemic.

Statistical Analysis. Results are expressed as mean±SEM. Differences between the experimental groups were analyzed using unpaired Student's t-test and analysis of variance (ANOVA) using SPSS statistical software (Chicago, IL). Differences were considered significant when $p<0.05$.

RESULTS

Islet Recoveries. The recovery of cryopreserved canine islets immediately following thawing and removal of the DMSO from a series of six individual experiments is outlined in Table VIII-1. Using a sucrose dilution the islet recovery from glass tubes was 81.5±4.2% (mean±SEM) (Table VIII-1). This was not significantly different from the recovery of islets with the DMSO removed using either the standard slow step or the modified slow step protocols (80±5.1% and 87±2.9% respectively). Following a period of post-thaw culture the islet recovery from Group 1 was significantly reduced as compared with the islet recovery from Group 3 (Table VIII-1). The percentage recovery for islets was 65.7±3% for Group 1 vs. 71.7±2.7% for Group 2 and 75.7±3.9% for Group 3 ($p<0.05$).

Islet recovery post-thaw from the freezer bags was 90±3.8% for Group 4 and 84±6.1% for Group 5 (p=ns) (Table VIII-1). However, after 48 hour tissue culture islet recovery was significantly higher when the cryoprotectant was removed using the automated modified slow step dilution (74.8±2.4%) as compared with the standard sucrose dilution (66.2±2.7%) ($p<0.05$, unpaired students t-test) (Table VIII-1).

Static Incubation. *In vitro* function of the frozen-thawed canine islets was assessed using a 2 hour 37°C static incubation in low, high and high glucose supplemented with 50 μm IBMX. Compared with non-frozen control islets, all groups of cryopreserved islets had a higher insulin output during the low glucose (Table VIII-2). However, only islets from Group 4 were significantly elevated ($p<0.05$ vs non-frozen controls, n=6 individual experiments run in duplicate) (Table VIII-2). During stimulation with high glucose (20 mmol/L) there was an 2-2.8 fold increase in insulin output from all frozen-thawed islets which was not significantly different than the non-frozen controls. When the islets were challenged with high glucose plus IBMX there was a further increase in insulin output in all groups, with the highest increase (9.6x) when the islets cryopreserved in the freezer bag where the DMSO was removed using the automated modified slow step protocol (Group 5) ($p<0.05$ vs. non-frozen controls) (Table VIII-2).

In vivo Graft Assessment. *In vivo* assessment of the frozen-thawed islets is outlined in Table VIII-3. No statistical difference in median graft survival or percent of animals remaining normoglycemic at 50 days post-transplant was observed in alloxan-induced diabetic nude mice transplanted with islets cryopreserved in glass tubes with the DMSO removed using the sucrose, slow step or modified slow step

protocol (Table VIII-3). Mice receiving cryopreserved islets frozen in the freezer bag with the DMSO removed using the modified slow step protocol showed 71% of the animals surviving at 50 days post-transplant which was equivalent to the survival rate for mice receiving fresh non-frozen islets (Table VIII-3) (p=ns).

Oral Glucose Tolerance Test. Figure VIII-4 demonstrates the response of euglycemic mice surviving 50 days post-transplant. Following glucose challenge we observed no difference in the functional response with all mice returning to baseline within 60 minutes post-glucose challenge.

Histology. Histological evaluation of the islet grafts removed from the animals at 50 days post-transplant revealed intact well-granulated islets under the kidney capsule from histological sections taken following nephrectomy at 50 days (Plates VIII-1, 2, 3).

DISCUSSION

Clinical islet transplantation offers great promise in the treatment of insulin dependent diabetes mellitus. Recently, there have been an increased number of patients who, following islet transplantation, have experienced periods of insulin independence (2-5). Of these successful cases several factors have been identified that increase the potential of achieving insulin independence post-transplant (5). Transplantation of an islet mass exceeding 6000 IE/kilogram body weight was one of the factors found in all patients that became insulin independent (5). Although methods of isolating islets are improving, the recovery of an islet mass that allows transplantation of greater than 6000 IE/kilogram body weight from a single cadaveric

donor is rarely achieved (26-28). The concept of using multiple donors to increase the transplantable islet mass is an option used by several clinical islet transplant programs worldwide. Many of the transplants have used cryopreserved islets to supplement freshly isolated islets.

Cryopreservation of isolated islets in a low temperature tissue bank is an effective method of storing and pooling tissue providing increased flexibility in clinical trials (1). Frozen tissue safely stored in liquid nitrogen permits easy transport between transplant centres (8), enables time for selection of tissue based on tissue type matching (1) and permits time for proper viability and microbiological testing (29).

Isolated islets have been successfully cryopreserved using several methods. A number of these approaches have evaluated different cryoprotectants, alternative methods used to add and remove the cryoprotectant and have varied cooling and thawing rates (30). In 1989, Rajotte et al. compared a variety of reported cryopreservation protocols and found that slow cooling (0.25°C/minute) to -40°C before plunging into liquid nitrogen in combination with rapid thawing (200°C/minute) resulted in similar *in vitro* function comparable to non-frozen controls (13). Coloumbe et al. confirmed these results in a transplant model where 3000 frozen-thawed rat islets normalized diabetic recipients and showed similar results to animals receiving freshly isolated islets (31). Rich et al. also confirmed that slow cooling and rapid thawing was superior to slow cooling to -70°C followed by slow warming at 50°C/minute (32). However, they observed impaired islet function following transplantation of 750 frozen-thawed islets as compared with non-

frozen controls (32). When the functional losses associated with the cryopreservation process were accounted for, islet function was comparable to non-frozen control isografts (32). Using the slow cooling to -40°C followed by plunging into liquid nitrogen in combination with rapid thawing, canine islets have been successfully autotransplanted (33, 34) and have been used in allograft studies (34). As well, human islets cryopreserved using these protocols have been used clinically (3, 5, 35).

Apart from Rajotte et al. (8), who compared a slow step dilution protocol to 0.75 M sucrose dilution protocol using rat islets, there has been little attention devoted to examining the methods used for the removal of the cryoprotectant. When evaluating these protocols for incorporation into a protocol which is capable of adding and removing the cryoprotectant within the freezer bag, the slow step protocol was not feasible because the total volume of dilution solution exceeded the capacity of the freezer bag (36). The sucrose dilution protocol was also not feasible because it requires multiple centrifugation steps and removal of the supernatant before the exposure to the 0.75 M sucrose solution. Therefore, a modified slow step protocol was developed which allows the removal of the cryoprotectant entirely within a single freezer bag. Initial experiments compared the modified slow step protocol to the standard methods of sucrose and slow step dilution following cryopreservation in glass tubes. The results showed equivalent recoveries of islets immediately post-thaw. Following tissue culture, however, the recovery of islets was significantly higher when the DMSO was diluted using either a slow step or modified slow step protocol as compared with DMSO removed using the sucrose dilution protocol.

Once the modified slow step protocol was shown to have comparable recoveries and functional viability, we automated this protocol and incorporated it into a cryopreservation protocol utilizing a single freezer bag. Cryopreservation of entire preparations of islets in a single freezer bag system offers several advantages to conventional methods of storage in glass tubes. The ability to add and remove the cryoprotectant within the freezer bag allows the potential to develop an automated system which would further enhance low temperature banking of islets.

When canine islets were cryopreserved and thawed using the automated modified slow step protocol in freezer bags we observed an approximately 15% loss of islets, however, the function of the remaining islets were comparable to non-frozen control islets during static incubation. The insulin output during exposure to high glucose plus IBMX was significantly elevated as compared with the non-frozen controls. Transplantation of frozen-thawed canine islets into alloxan-induced diabetic nude mice were able to reverse and normalize the blood glucose with graft survival results comparable to the success rate observed using non-frozen control canine islets. An oral glucose tolerance test on animals remaining normoglycemic >50 days post-transplant showed no differences between the experimental groups with the results comparable to those observed for non-transplanted age matched control mice. This study evaluated an automated modified slow step method for the removal of the DMSO following cryopreservation. The advantage of this method is the potential to develop a protocol that enables the entire process to be completed within the freezer bag. This has several advantages including the development of an automated computer controlled protocol that is capable of adding and removing the solutions

without exposing the tissue to ambient air, thereby reducing the risk of microbial contamination. Our results using canine islets indicate that the modified slow step method allows for an increased recovery of islets following post-cryopreservation tissue culture for islets cryopreserved in either glass tubes or in freezer bags. *In vitro* and *in vivo* viability was comparable to non-frozen control islets.

CONCLUSION

The ability to develop a cryopreservation protocol that allows the automated addition and removal of the cryoprotectant offers several advantages to current methods of islet cryopreservation.

These data suggest that a modified slow stepwise addition of isotonic media does not compromise islets recovery, survival in culture and functional viability using both static incubation and *in vivo* transplantation. An automated protocol enables the controlled application of this technology and further advances the low temperature tissue banking of isolated islets.

Table VIII-1:

Islet recovery following freeze-thaw and 48 hour *in vitro* tissue culture.

Experimental Group	% recovery after thawing[a]	% recovery after culture
Islets cryopreserved in glass tubes		
1) Sucrose dilution	81.5±4.2	65.7±3.0
2) Standard slow step	80.0±5.1	71.7±2.7
3) Modified slow step	87.0±2.9	75.7±3.9[b]
Islets cryopreserved in freezer bag		
4) Sucrose dilution	90.0±3.8	66.2±2.7
5) Modified slow step	84.0±6.1	74.8±2.4[b]

[a] mean±SEM

[b] $p<0.05$, unpaired t-test vs. sucrose dilution protocol

Table VIII-2:

Insulin response of frozen-thawed islets during static incubation.

Experimental Group[a]	Insulin Secretion (μU/IE/hr)		
	2.8 mmol/L	20	20 + IBMX
Islets cryopreserved in glass tubes			
1) Sucrose dilution	3.2±0.6	7.1±0.6	17.9±1.4
2) Standard slow step	2.8±0.4	6.3±0.4	16.9±1.0
3) Modified slow step	3.0±0.5	6.5±0.2	19.0±1.9
Islets cryopreserved in freezer bag			
4) Sucrose dilution	3.4±0.4[b]	6.7±0.8	21.2±1.7
5) Modified slow step	2.4±1.1	6.8±0.6	23.0±1.4[b]
Non-frozen controls	2.1±0.4	6.4±0.6	18.2±1.0

[a] mean±SEM

[b] $p<0.05$, unpaired t-test vs. non-frozen controls

Table VIII-3:

***In vivo* function of alloxan induced diabetic nude mice transplanted with frozen-thawed islets.**

Experimental Group	Days of normoglycemia	Percent normoglycemia at 50 days
Islets cryopreserved in glass tubes		
1) Sucrose dilution	0x2,36,>50x4	57%
2) Standard slow step	0x2,11,>50x4	57%
3) Modified slow step	0,11,17,>50x4	57%
Islets cryopreserved in freezer bag		
1) Sucrose dilution	0,14,>50x4	57%
2) Modified slow step	11,28,>50x5	71%
Non-frozen controls	0x2,>50x5	71%
Non-transplanted diabetic controls	4,8x2,10x2	0

Figure VIII-1:

Protocol for the rapid thawing and removal of the cryoprotectant using a 30 minute exposure to 0.75 M sucrose solution followed by serial dilution with Thaw Media.

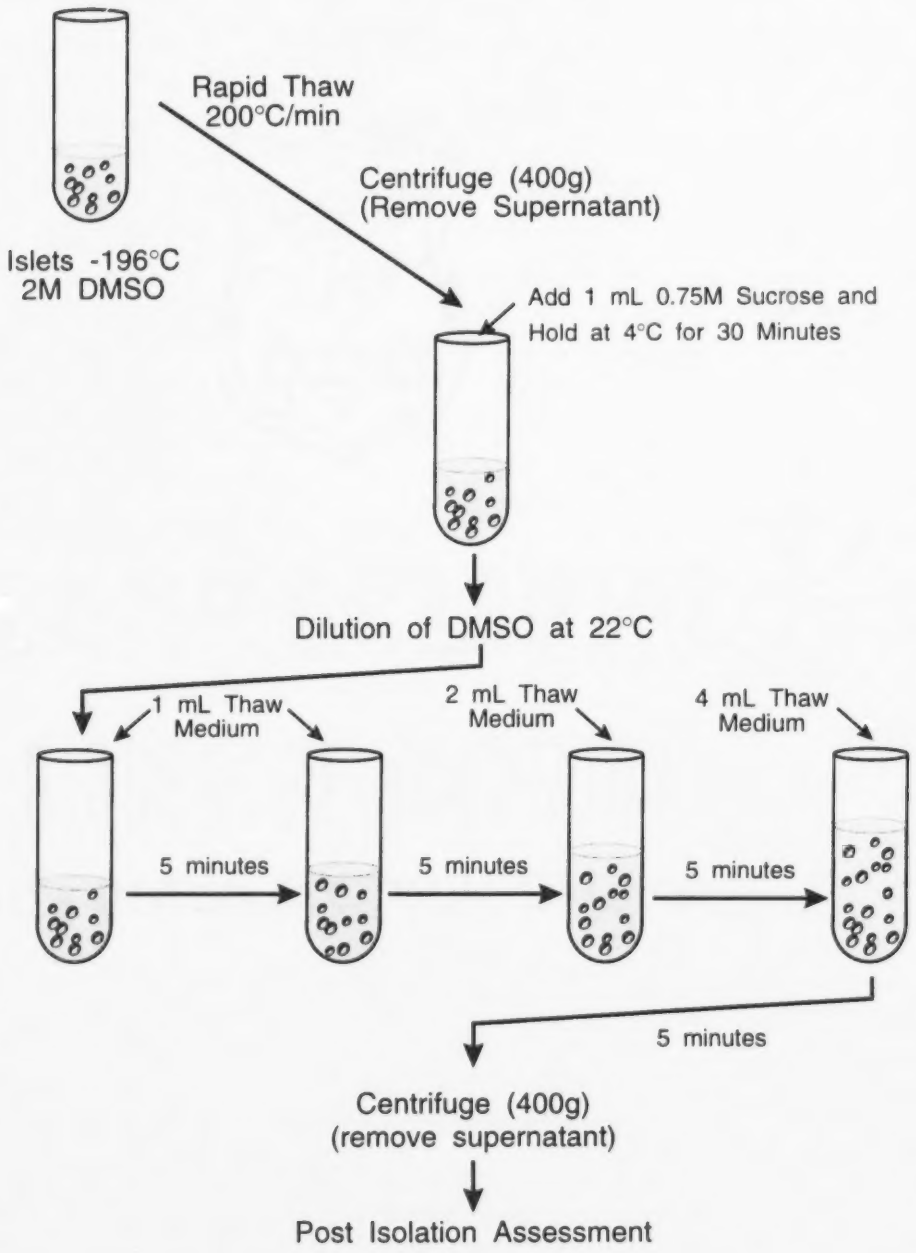

Figure VIII-2:

Protocol for the rapid thawing and removal of the cryoprotectant using slow step dilution.

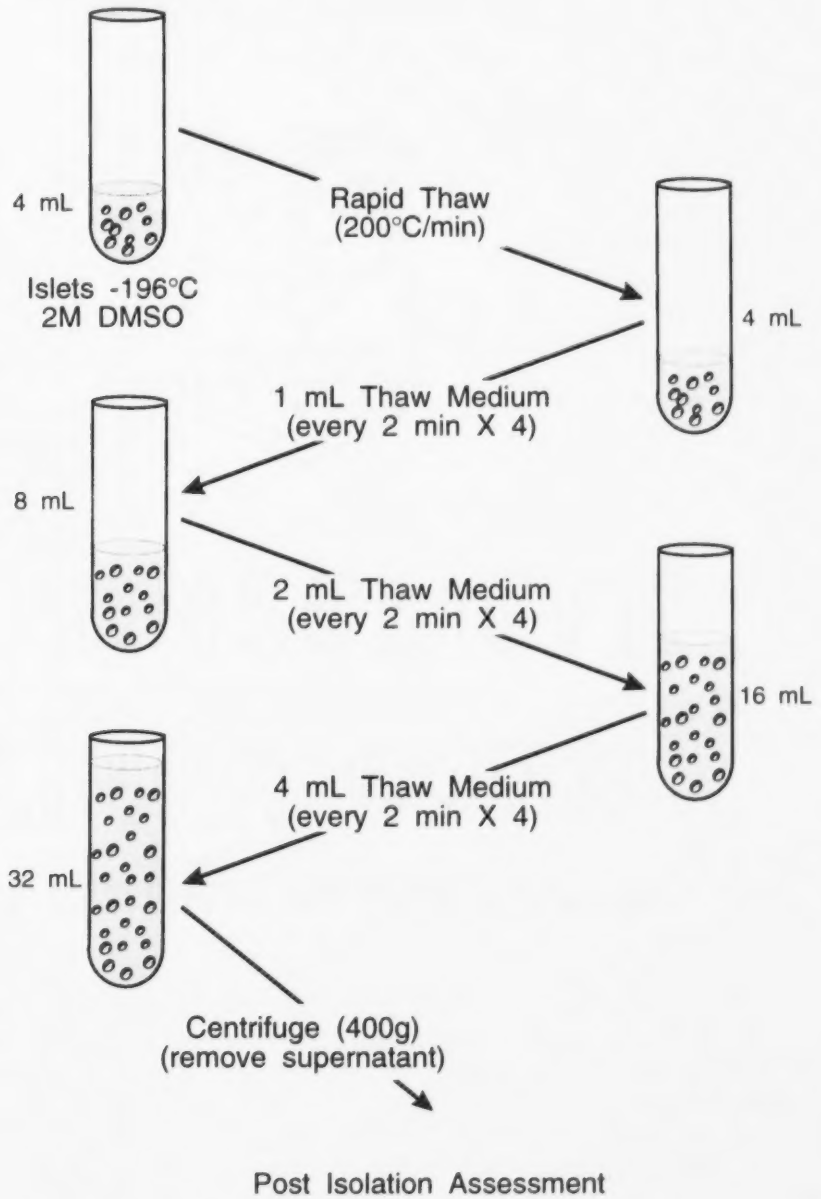

Figure VIII-3:

Protocol for the rapid thawing and removal of the cryoprotectant using a modified slow step dilution.

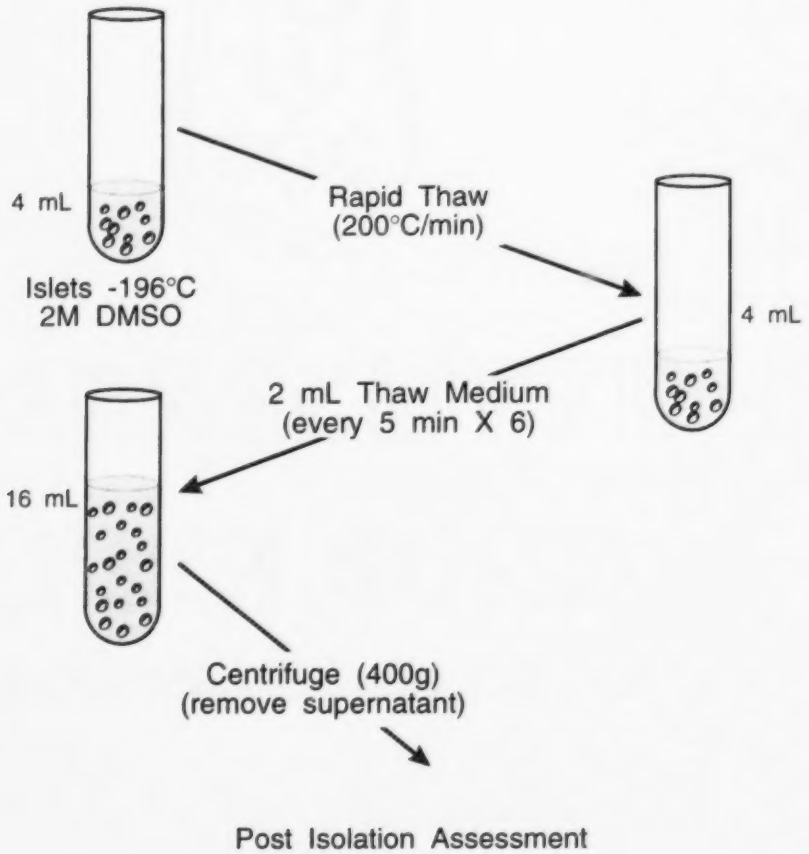

Figure VIII-4:

Oral glucose tolerance test of alloxan-induced nude mice transplanted with canine islets and monitored for 50 days.

A. Mice receiving freshly isolated islets (open triangle), or islets cryopreserved in glass tubes with DMSO removed using sucrose dilution (closed box), standard slow step (closed circle) or the modified slow step protocol (closed diamond).

B. Mice receiving freshly isolated islets (open triangle), or islets cryopreserved in freezer bags with DMSO removed using sucrose dilution (closed box) or modified slow step protocol (closed diamond).

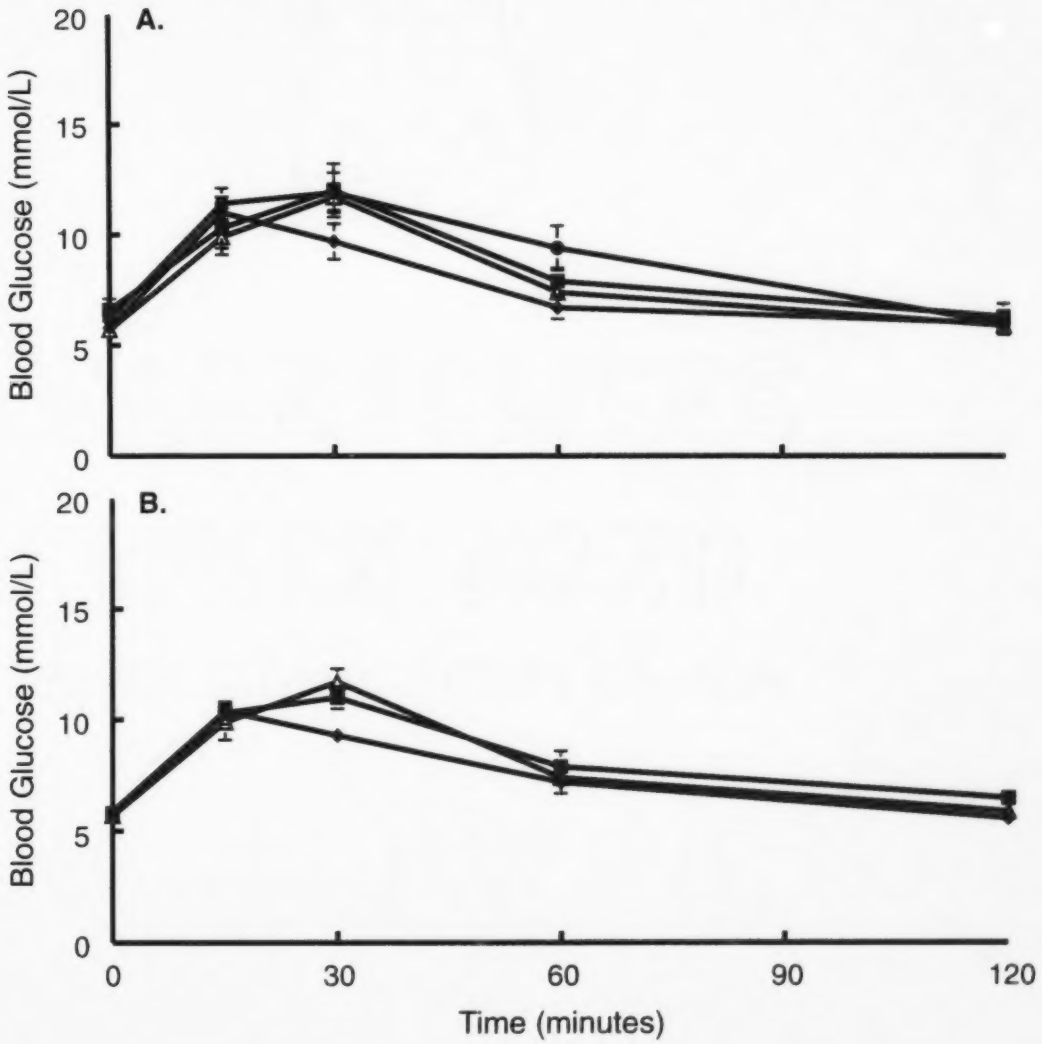

Plate VIII-1:

Photomicrograph of freshly isolated canine islets placed under the renal capsule of an alloxan-induced diabetic nude mouse. Aldehyde-fuschin stained section showing well granulated beta cells. (original magnification x 200)

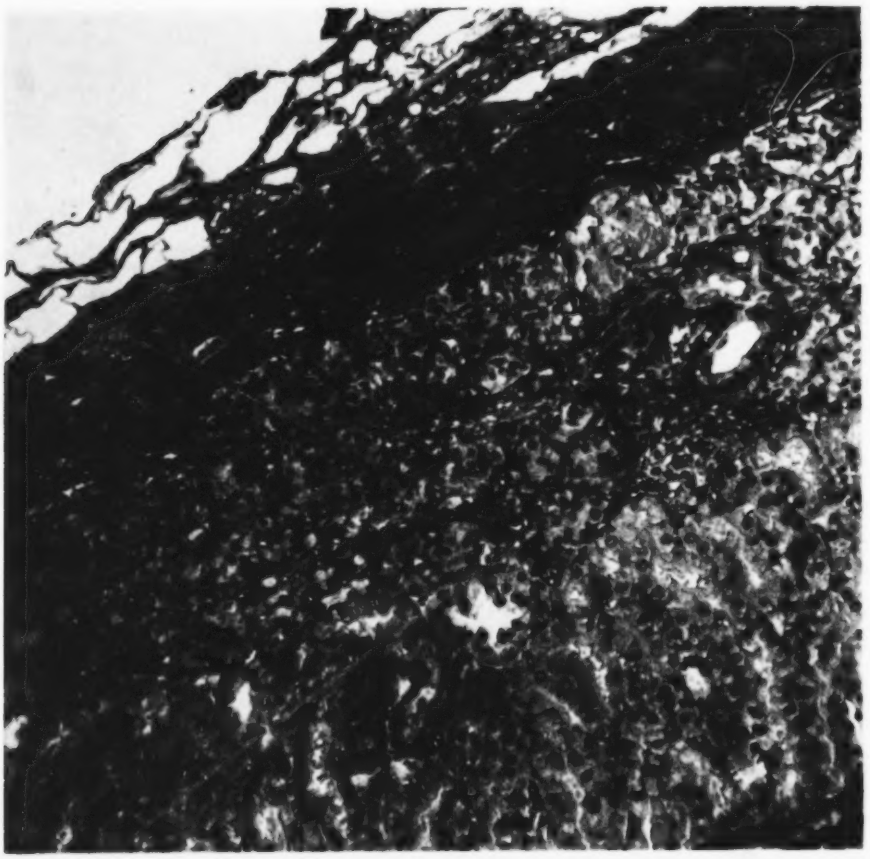

Plate VIII-2:

Photomicrograph of frozen-thawed canine islets cryopreserved in the freezer bag and the DMSO removed using the standard sucrose dilution protocol. Islets were placed under the renal capsule of an alloxan-induced diabetic nude mouse. Aldehyde-fuschin stained section showing well granulated beta cells. (original magnification x 200)

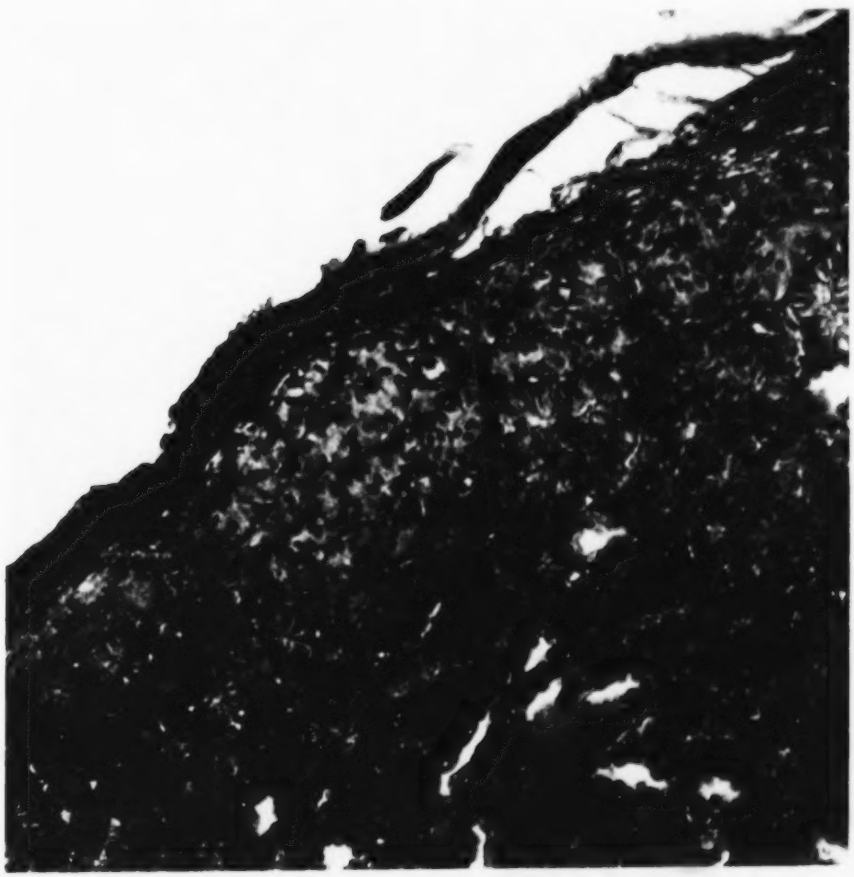

Plate VIII-3:

Photomicrograph of frozen-thawed canine islets cryopreserved in the freezer bag and the DMSO removed using the modified slow step protocol. Islets were placed under the renal capsule of an alloxan-induced diabetic nude mouse. Aldehyde-fuschin stained section showing well granulated beta cells. (original magnification x 200)

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IX

GENERAL DISCUSSION AND CONCLUSIONS

DISCUSSION

Clinical islet transplantation offers great potential in the treatment of diabetes. Evaluation of recent clinical islet transplants reported to the International Islet Transplant Registry identified four common characteristics in patients that were able to become insulin independent following islet transplantation. These include: isolation of islets from the cadaveric donor within eight hours following procurement, transplantation of islets into the liver via the portal vein, induction immunosuppression therapy consisting of T cell antibodies and the transplantation of an islet mass exceeding 6000 IE/kilogram body weight (1, 2). When all four criteria were met, the proportion of patients that were insulin independent at one year follow-up was 28% (2). If islet transplantation is to become an effective and viable option in the treatment of diabetes the post-transplant success must improve. It was the aim of this thesis to address two key issues limiting clinical islet transplantation. The first is the lack of consistency and reliability in the ability to isolate large numbers of human islets. The second issue addresses the lack of effective methods for the large-scale low temperature banking of islets.

Superior Human Pancreatic Procurement and Islet Isolation. Chapter II evaluated several factors in the multi-organ donor that could potentially affect the recovery and function of islets. Recently, there have been several studies that have evaluated donor-related variables in regards to the recovery and function of islets

(3-6). These studies consisted of relatively small sample sizes and the statistical evaluations compared one factor to islet recovery. To expand upon these observations with a strong study design, we used multivariate analysis to examine the interrelationship of related donor variables. Chapter II retrospectively analyzed 153 isolations evaluated using logistic and multiple regression analysis. Donor age, body mass index and procurement of the pancreas by our local procurement team were found to be positively correlated with islet isolation success. In contrast, donor hyperglycemia, frequency and duration of cardiac arrest and the increased duration of cold storage before islet isolation were found to adversely affect the recovery of islets. Using this combination of factors, the model had an accuracy of predicting success of 85%. This was considerably higher than the accuracy of the donor model described by Benhamou et al. which was correct in predicting successful isolations in 66.2% of the cases (6).

Multiple regression analysis using the post-purification islet recovery as outcome variable confirmed many of the factors identified using logistic regression analysis. In addition, increased duration of collagenase digestion time was identified as a factor that significantly affected post-isolation islet recovery. Additionally, increased processed pancreas weight had a negative correlation with islet isolation success. This suggests that the automated dissociation chamber may have been overloaded leading to a suboptimal dissociation. As well, the concentration in the dissociation chamber may have become saturated.

The effect of donor-related variables on islet function was evaluated using the stimulation index from the *in vitro* viability assessment of the islets. Grouping donors

into several age categories and evaluating islet function showed that the insulin secretory capabilities from the group of donors that was >50 years old were significantly reduced as compared with the islet function from young 2.5-18 year-old donors. Multiple regression analysis using *in vitro* islet function as the outcome variable identified donors with elevated minimum blood glucose and pancreases with increased duration of cold storage before islet isolation as factors which negatively affected *in vitro* islet function.

This review of parameters from a large series of isolations and the multivariate analysis of several key variables suggest an improved approach to the prediction of successful islet isolation from human pancreases. Selection of suitable pancreases for processing will improve the consistency and reliability of human islet isolations. The potential impact of these findings is that we can now accurately predict whether or not an isolation will yield >100,000 islets. This has financial implications and allows one to focus efforts on isolations that will have a higher potential for success, as determined by the statistical model. Although no difference was found between the four lots of collagenase used in this evaluation, selection of an optimal lot of collagenase is critical to the isolation outcome. Future analysis of donor-related variables from isolations using a stable and consistent enzyme preparation such as Liberase (7) will perhaps provide an even more accurate model.

Data from Chapter II identified the duration of cold storage as a factor that significantly affects both islet recovery and *in vitro* islet function. The research project in Chapter III further examined the issue of cold storage and demonstrated that the maximal duration of cold ischemia tolerated by the human pancreas before

islet isolation is 16 hours. Evaluation of both post-digestion and post-purification islet recovery provided a more accurate assessment of the effective of cold storage on the ability to dissociate the pancreas and purify the islets. When viability was assessed using *in vitro* glucose perifusion, islets isolated from the group of pancreases with greater than 16 hours of cold storage were unresponsive to high glucose, with a significant fall in the calculated stimulation index. This indicates that, in addition to a significant fall off of islet recovery, a deterioration in function occurs. The implications of this finding are valuable to processing centres receiving organs from distant procurement locations. Decisions to process pancreases can be made with confidence based on these findings. Direction of future research may involve improving the maximal duration of cold storage by the development of improved preservation solutions and the testing of new techniques which relate an isolation protocol based on the duration of cold storage. Additionally, the finding that there is a decrease in functional viability of islets with increased duration of cold storage in UW solution is an important observation and is supported by previous preservation studies with Euro-Collins solution (8) and data from the Islet Transplant Registry (1). Although analysis from the Islet Transplant Registry was performed on only 16 cases with cold ischemia >8 hours, the observation that no patient receiving islets from a pancreas with >8 hours cold storage is important. Future evaluations of comparing *in vitro* and *in vivo* islet function from islets isolated from human pancreases with prolonged cold storage should clarify this key issue.

Procurement of the human pancreas is critical to subsequent islet recovery. Previous studies from both rodent (9, 10) and canine models (11) suggest a

detrimental impact on islet recovery and function when the pancreas is removed following *in situ* vascular flushing with UW preservation solution. The majority of pancreases that are procured for human islet isolation are procured following vascular flushing with UW solution (12). Chapter IV compared two methods of human pancreas procurement from a consecutive series of adult multi-organ donors. This evaluation determined that large numbers of viable islets can be recovered following the use of either 1) *in situ* vascular perfusion with UW organ preservation solution followed by total pancreatectomy or 2) following a subtotal pancreatectomy procedure before *in situ* vascular flushing. The total pancreatectomy following *in situ* vascular flushing procedure offers decreased overall operating time and is technically less complicated to perform. These advantages in conjunction with the results of this study led to a change in our present procurement protocol.

Recent findings suggest that further attention must be given to the fact that UW solution inhibits specific factors in the collagenase (13). In our evaluation a significantly longer collagenase digestion time from organs procured following UW flushing was noted despite augmenting the collagenase dose. Future experiments which remove the UW preservation solution before processing may provide evidence of a reduction in these inhibitory effects.

Current methods of human islet isolation requires a certain level of expertise. In an attempt to provide a more standard and consistent method of islet isolation, Chapter V described the development and evaluation of a computer-based automated cell extraction system (ACES). Initial development focused on the optimal system tubing configuration and evaluation of a novel particle collection

system. Digested pancreatic tissue are effectively separated and collected along a flat length of PVC tubing. This collection system, termed sluice, eliminates the need for several centrifugation steps during tissue recombination and enables all equipment in the isolation process to be encompassed within a single disposable set. With complete computer control and monitoring of the dissociation and recombination phases, the ACES system allows development of an automated procedure and can provide extensive history files of each isolation. Experimental data shows that an entirely automated pancreatic islet extraction system can result in effective canine islet recovery without compromising islet yields and viability. Future evaluation of the ACES system with human pancreas dissociation may lead to the development of a protocol that allows consistent and reliable islet recoveries from the human pancreas.

Improved ability to identify optimal cadaveric donors, knowledge of acceptable durations of cold storage, standardization of methods used to procure the pancreas and the development of a means to effectively separate large numbers of viable islets improves the consistency and reliability; facilitating improved clinical trials.

Directions in Low Temperature Banking of Islets A second important issue limiting clinical trials are the less than optimal methods of low temperature banking of human islet preparations. This issue was addressed by first examining quality control issues in the isolation and low temperature preservation of islets. Development and incorporation of improved methods to cryopreserve large numbers of islets within a freezer bags enabled more effective banking of islets.

The issue of quality control and the maintenance of microbiological sterility during islet isolation, *in vitro* tissue culture and during low temperature storage in liquid nitrogen was evaluated in Chapter VI. The incidence of microbiological contamination from a series of 47 preparations of cryopreserved human islets was 27%. It was not possible to ascertain the source of the contaminants, as the cultures for microbiological assessment were taken only following cryopreservation. A total of 70 consecutive human pancreases were processed with microbiological samples removed at multiple steps during isolation and cryopreservation to determine the incidence and source of the contamination. Following procurement, a total of 19% of the pancreas received for islet processing were microbiologically contaminated with an increased proportion of these (26%) occurring in organs received from distant centres. The incidence is lower than what has been previously reported from the St. Louis and Pittsburgh islet transplant programs (14, 15). For locally procured pancreases, no difference in microbiological contamination was observed from pancreases removed before or following *in situ* vascular flushing. The incidence of microbiological contamination tends to decrease during processing.

Evaluation of microbiological contamination during low temperature banking of islets has not been previously reported. Chapter VI observed that preparations of islets cryopreserved in glass tubes remain sterile despite liquid nitrogen seeping into the tubes. These findings are of vital importance as they indicate where breaks in sterile technique occur. Therefore, it is necessary for centres to actively monitor their isolation and banking practices in order to prevent the transplantation of contaminated islets into immunosuppressed patients.

Current methods of cryopreservation of human islets are both labour-intensive and cumbersome due to the practice of placing small aliquots of islets per individual glass tube (17, 18). In an attempt to improve the quality of cryopreserved islets to be used for transplantation and to develop methods that will facilitate effective low temperature banking of islets, Chapter VII described the evaluation of a freezer bag system for the cryopreservation of islets. Chapter VIII described the development of an automated cryopreservation protocol using the freezer bag system which permits the addition and removal of the cryoprotectant within the freezer bag.

Recovery and functional viability of canine islets cryopreserved in freezer bags was equivalent to islets cryopreserved in the standard glass tubes. Bulk cryopreservation of islets in a single freezer bag has several advantages, including: 1) the ability to freeze an entire preparation of islets in a single bag is simpler and less prone to inadvertent mixing of tubes, 2) it is more cost effective as it is less labour-intensive and requires less storage space, and 3) by freezing in a single sealed bag there is less opportunity for microbiological contamination, and 4) an automated protocol can be developed for the automated addition and removal of the cryoprotectant.

The development of an automated protocol for the addition and removal of the cryoprotectant within the freezer bag would is a significant improvement to current methods of islet cryopreservation as the entire process can be accomplished automatically without exposing the tissue to ambient air, thus decreasing the chance of microbiological contamination. Chapter VIII evaluated a cryopreservation protocol that adapted the slow step dilution protocol (19, 20) for the removal of the

cryoprotectant post-thaw. The modified slow step dilution protocol enables the slow stepwise dilution of the cryoprotectant to be performed within the freezer bag. Experiments using cryopreserved canine islets indicate that the modified slow step protocol allows equivalent recovery and functional viability when compared with islets cryopreserved in freezer bags and the DMSO removed using the sucrose dilution protocol. The standard procedure for the sucrose dilution protocol is to centrifuge and remove the supernatant. The centrifugation step immediately following thawing of the islets may cause increased susceptibility to damage. These new developments constitute significant advancements in the cryopreservation of human islets and will improve the methods of low temperature tissue banking of islets facilitating improved clinical islet transplant trials.

CONCLUSIONS

The research projects included in this thesis have made significant advancements in the field of islet isolation and cryopreservation. Significant findings include:

I. an optimized model for predicting the success of islet isolations from individual human organ donor pancreases,

II. the identification of factors in multi-organ cadaveric donors using multivariate logistic stepwise regression analysis that were positively correlated with islet recovery (donor age, body mass index and surgical procurement team) and factors that were negatively correlated with isolation success (donor hyperglycemia, frequency and duration of cardiac arrest and the duration of cold storage),

III. that the islet isolation success rate was highest from pancreases harvested from older donors; however, these islets showed significantly reduced insulin secretion capabilities as compared with islets from juvenile donors,

IV. that a significantly decreased recovery and impaired *in vitro* viability of islets occurs in adult cadaveric donor pancreases stored for periods of >16 hours in UW solution,

V. the ability to recover large numbers of viable islets from cadaveric human pancreases utilizing *in situ* vascular flushing with UW solution before total pancreatectomy or a subtotal pancreatectomy procedure,

VI. that the development of an entirely automated isolation system using the canine model can result in effective recovery of isolated islets without compromising islet yields and functional viability,

VII. the identification of steps in the isolation process where microbiological contaminants occur,

VIII. that the low temperature banking of isolated islets does not introduce new microbiological contaminants into preparations during storage in liquid nitrogen,

IX. that careful surveillance of multiple steps in the isolation and cryopreservation of islets ensures effective quality control,

X. that cryopreservation of isolated islets in a single freezer bag is an effective method of bulk low temperature storage of islets,

XI. that the removal of the DMSO can be effectively achieved using a modified slow step dilution protocol resulting in increased islet recovery following a period of post-cryopreservation tissue culture,

XII. that incorporation of the modified slow step dilution protocol allows the development of an automated protocol for the bulk cryopreservation of islets in a freezer bag system.

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